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14. ABSTRACT

Food Allergies are a common pediatric condition and has been reported up to 1 in 4 children and nearly 3 million children reporting a food reaction in 2007 based on the latest Center for Disease Control report in Nov 2008. Food Allergies can be divided into several types of reactions: urticarial, anaphylaxis, respiratory and gastrointestinal. The reactions can be further divided based on mechanism of reaction into IgE and non-IgE. In conjunction with the Center for Applied Genomics at The Children's Hospital of Philadelphia, we used genome wide association (GWA) studies whole exon sequencing to identify many new genes in food allergy. We identified TSLP as a region of interest in Eosinophilic Esophagitis. We were able to confirm the importance of the region in human studies finding direct correlation with eosinophils and TSLP. In addition, we also identified two additional regions that play a role in food allergy. These genes were also identified in mechanistic studies identifying new pathways for treatment.

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INTRODUCTION:

Approximately 25% of the United States population believes that they have an allergic reaction to foods. The prevalence of food allergies (FA) have increased 18% in the last 10 years and over 9500 hospital admission for FA were noted from 2004-06 making it an urgent medical need. Finally, many FA are life-long and are significant risk for adults including potentially military personnel without any significant potential treatment available except for avoidance. FAs are a broad category and are part of the larger adverse food reaction, which is any reaction to food regardless of the pathophysiology. The food reactions are split into immunologic and non-immunologic entities. Non-immune reactions include jitteriness from caffeine or metabolic disorders such as lactose deficiency. The immune reactions are what physicians consider **FA**. Immune reactions are further divided into IgE- and non-IgE-mediated reactions. There are also food induced immune responses such as celiac disease, which there is an autoimmune reaction to gliadin, a component of wheat, rye and barley. IgE-mediated reactions are the classical presentations of FA such as hives or anaphylaxis after eating the offending food antigen. Non-IgE-mediated FA have been more recently described and include food protein-induced enterocolitis syndrome. Other food reactions such as atopic dermatitis and eosinophilic esophagitis (EoE) reactions are considered mixed IgE and non-IgE. To further complicate the scenario, patients often have a combination of different types of FA such as anaphylaxis to one food and atopic dermatitis to another

BODY:

We completed the study and statement of works as outlined. Our specific aims were

Specific Aim 1. To perform a whole genome scan to test for association of EoE and Food Allergy with SNPs, SNP haplotypes or copy number variations using high-throughput tag-SNP arrays.

Specific Aim 2. To determine the expression of candidate genes in Esophageal tissue or peripheral blood monocytes

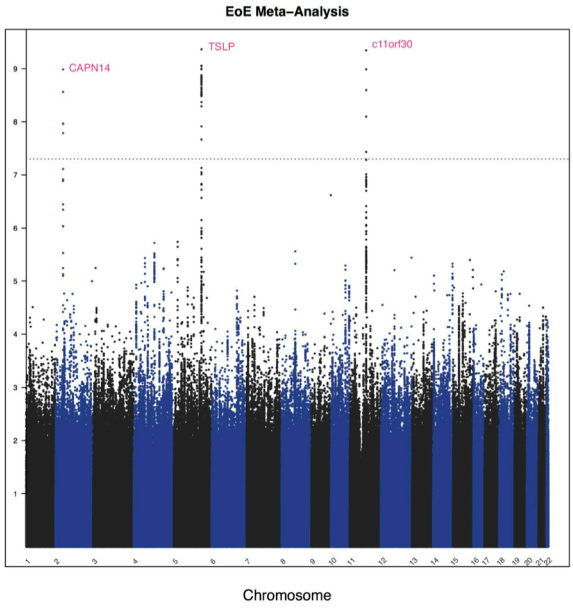
Subaim 2.1: Examine candidate genes for IgE mediated food allergies in PBMC
Subaim 2.2: Examine candidate genes for EoE in esophageal tissue and PBMC

The statement of work included collection of DNA samples from The Children's Hospital of Philadelphia (CHOP) and outside organizations. We collected/received samples from CHOP, Cincinnati Children's Hospital, Stanford University, Univ. of Colorado and UC San Diego. Other items in the statement of work included Genome Wide Association analysis (GWAS) of the CHOP cohort for both Eosinophilic Esophagitis and IgE mediated food allergy. These analyses were completed and results are indicated below.

In addition, the statement of work entitled replication cohort for Eosinophilic Esophagitis using samples from other institutions. These data was also completed and outlined below.

GWAS of CHOP cohort and using other institutions replicated the TSLP gene loci and 2 new regions (**c11orf30 and CAPN14)** based on SNP variants in EoE (Figure 1).

Figure 1: Manhattan plot of the EoE meta-analysis, -log10 Pvals on the y-axis plotted against ascending physical position on the x-axis. The dotted red line represents the genome-wide significance threshold Pval ≥ $5x10^{-8}$.



Examination of esophageal samples and their protein expression confirmed these proteins to be important for food allergy. We found that TSLP expression was altered in EoE patients compared to control. We also examined peripheral blood in patients with Eosinophilic Esophagitis examining risk alleles and proteins. (Figure 6; Noti et al., Nature Medicine) We found that TSLP and basophils were required in murine model of EoE (Figure 2-5, Noti et al, Nature Medicine). These same cells and proteins were increased in our patients with EoE compared to the healthy controls in both adult and pediatric patients (See Figure 6, Noti et al, Nature Medicine).

To further analysis and characterize our food allergy patients, we develop Eosinophilic Esophagitis Cell line (Muir et al., Exp Cell Research). We found that stimulation of primary fetal esophageal fibroblasts (FEF3) with conditioned media (CEM) from esophageal epithelial cells (EPC2-hTERT), primed FEF3 cells to secrete IL-1b and TNFa, but not TGFb (Figure 1). To determine whether these cytokines signaled in a paracrine fashion to esophageal epithelial cells, FEF3 cells were stimulated with CEM, followed by transfer of this fibroblast conditioned media (FCM) to EPC2-hTERT cells. Epithelial FCM stimulation increased expression of mesenchymal markers and reduced E-cadherin expression, features of EMT which were TNFa and IL-1b-dependent (Figure 3). Using organotypic culture models, primary EoE epithelial cells exhibited features of EMT compared to non-EoE cells (Figure 4), corresponding to patterns of EMT in native biopsies (Figure 5). This work demonstrates a potential mechanism of fibrosis, which is one of the most important symptoms of EoE-dysphagia and strictures.

We were not able to identify any risk allele for IgE mediated food allergy in a cohort of >1400 patients at CHOP from GWAS analysis. However, we also examined biomarkers as part of the Statement of work. Seventy-three patients from 23 families were recruited. Culprit foods included milk (n -20), egg (n -10), and peanut (n-6) for food allergy and milk (n-20) and egg (n- 7) for food-triggered AD. Odds of having had a self-reported related food allergy or food-triggered AD reaction significantly increased witha higher number of detectable microarray components to that food. Ara h 1, Ara h 2, and Ara h 6 were individually associated with reported peanut allergy, and Bos d 4 was individually associated with reported milk allergy. The number of egg components significantly increased the odds of having related food triggered AD. To summarize, we found that

increased number of specific IgE to individual epitopes of an allergen correlated with food allergy to a higher degree than any one specific allergen. These results published in Annuals of Allergy Asthma and Immunology in 2013.

KEY RESEARCH ACCOMPLISHMENTS:

Research partially funded by this project

- Completed Genome Wide Array analysis of 1400 patients with Food Allergy
- Completed Whole Exon of 20 family trios with Food Allergy
- Completed Genome Wide Array Analysis of 1000 patients with Eosinophilic Esophagitis (Figure 1)
- Identified 2 novel regions for Eosinophilic Esophagitis (Figure 1)
- Confirmed TSLP loci for Eosinophilic Esophagitis by GWAS Analysis (Figure 1).

REPORTABLE OUTCOMES:

Outcome that were partially or full funded by this research

- Eosinophilic Esophagitis Cell line
- Developed murine model of Eosinophilic Esophagitis
- Additional funding based on this research from
  1. *Sensitization to allergens and progression to fibrosis are different in juvenile and adult mouse models of EoE funded by Joint Penn-CHOP Center for Digestive, Liver and Pancreatic Medicine, funded by*
  2. Role of TSLP and Basophils in Adult and Pediatric Eosinophilic Esophagitis, Pilot Grant from Institute for Translational Medicine and Therapeutics, Grant Number UL1RR024134 from NIH

CONCLUSION:

During the project, we were able to demonstrate the important genetic risk factors in food allergy and particularly Eosinophilic Esophagitis (EoE). In EoE, we demonstrate the genetic region of TSLP is a risk factor for developing EoE. In the next step of the project, we demonstrate changes in protein and mRNA expression in patients with EoE compared to the control (non-affected patients). We further demonstrated the importance of TSLP in the development of EoE in a murine model. Deleting TSLP gene by genetic knockouts or selective antibodies also eliminate the development of EoE in our murine model. We were also able show depletion of basophils also prevented the development of murine EoE.

The next step is a therapeutic trial of anti-TSLP in treating EoE. This work will need to be funded by future projects or pharmaceutical companies.

For the treatment of IgE-mediated food allergy, we have shown that compenent testing

maybe a better diagnostic test for food allergy than standard skin testing or specific IgE. This knowledge will allow for better and more accurate diagnosis of food allergy.

An important by-product of this work was development of EoE cell line. This cell line will enable more rapid screening of potential agents for the treatment of EoE.

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APPENDICES:

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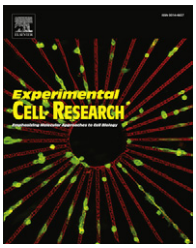

Research Article

Esophageal epithelial and mesenchymal cross-talk leads to features of epithelial to mesenchymal transition *in vitro*

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ABSTRACT

Background: Esophageal fibrosis is a complication of eosinophilic esophagitis (EoE) which has been attributed to both subepithelial fibrosis and to epithelial to mesenchymal transition (EMT), a process by which epithelial cells acquire mesenchymal features. Common to both causes of EoE-fibrosis is the notion that granulocyte-derived TGF-β, induces myofibroblast differentiation of the target cell. To date, the role of esophageal epithelial cells as effector cells in esophageal fibrosis has never been explored. Herein, we investigated consequences of cross-talk between esophageal epithelial cells and fibroblasts, and identified profibrotic cytokines which influence the development of EMT *in vitro*.

Methods and results: Stimulation of primary fetal esophageal fibroblasts (FEF3) with conditioned media (CEM) from esophageal epithelial cells (EPC2-hTERT), primed FEF3 cells to secrete IL-1β and TNFα, but not TGFβ. To determine whether these cytokines signaled in a paracrine fashion to esophageal epithelial cells, FEF3 cells were stimulated with CEM, followed by transfer of this fibroblast conditioned media (FCM) to EPC2-hTERT cells. Epithelial FCM stimulation increased expression of mesenchymal markers and reduced E-cadherin expression, features of EMT which were TNFα and IL-1β-dependent. Using organotypic culture models, primary EoE epithelial cells exhibited features of EMT compared to non-EoE cells, corresponding to patterns of EMT in native biopsies.

Conclusions: Esophageal epithelial cell and fibroblast cross-talk contributes to esophageal fibrosis. Our results suggest that features of EMT can develop independent of TGF-β and granulocytes, which may have important implications in treatment of EoE.

Abbreviations: EoE, eosinophilic esophagitis; EMT, epithelial to mesenchymal transition; IL-1β, interleukin 1-beta; TNFα, tumor necrosis factor alpha; TGFβ, transforming growth factor beta; αSMA, alpha smooth muscle actin; CEM, conditioned epithelial media; FCM, fibroblast conditioned media; OTC, organotypic cell culture

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Introduction

Eosinophilic esophagitis (EoE) is a chronic allergic disease affecting 4 in 10,000 children [1] and adults, characterized by eosinophilic infiltrates of the esophageal mucosa. In older children and adults, the most problematic complication of EoE is the development of esophageal fibrosis leading to dysphagia and esophageal food bolus impactions. The precise etiology of EoE-associated fibrosis remains unknown.

Fibrosis is defined as the inappropriate deposition of extracellular matrix (ECM), leading to deformation of the parenchyma. It is widely believed that stimulation with pro-fibrotic cytokines activates fibroblasts to acquire the activated phenotype of myofibroblasts, morphologic intermediates between fibroblasts and smooth muscle cells which synthesize ECM components including collagen, α-smooth muscle actin (αSMA), fibronectin, and proteoglycans. Although local fibroblasts are considered to be the most common myofibroblast progenitors, myofibroblasts have also been shown to originate from bone marrow-derived fibrocytes [2–4] and smooth muscle cells [5]. In addition, epithelial cells can acquire a myofibroblast characteristics and lose epithelial cell features [6] via epithelial to mesenchymal transition (EMT) [7]. In EMT, epithelial cells gain contractile and cytoskeleton proteins found in myofibroblasts while losing their characteristic tight junction and adhesion proteins.

Others have recently shown that EoE-associated fibrosis occurs through several mechanisms, including EMT. Aceves et al. showed that esophageal biopsies from EoE patients exhibit increased subepithelial collagen deposition compared to biopsies from control patients and patients with gastroesophageal reflux disease [8] suggesting that activation of fibroblasts within the subepithelium contributes to EoE fibrosis. In contrast, Kagalwalla et al. recently demonstrated that esophageal biopsies from pediatric EoE subjects exhibit features of EMT, characterized by increased expression of the mesenchymal marker vimentin and decreased expression of the epithelial marker cytokeratin within the epithelial compartment [9]. Interestingly, Kagalwalla et al. also observed a correlation between EMT scores and subepithelial fibrosis in pediatric EoE biopsies, indicating that the two processes are not mutually exclusive. In addition, these investigators also showed that features of EMT could be induced *in vitro*, through stimulation of the HET-1A esophageal epithelial cell line with the profibrotic cytokine, transforming growth factor-β (TGF-β), consistent with findings of Ohashi et al., who also showed that TGF-β stimulation induced EMT in the EPC2-hTERT esophageal epithelial cell line [10].

TGF-β is known as a prototypical profibrotic cytokine in many models of fibrosis [11–13]. Consistent with this notion, both Aceves et al. and Kagalwalla et al. have suggested that TGF-β is necessary for myofibroblast activation in the context of EoE-associated fibrosis. This assumption is supported by the work of others, who have previously shown that TGF-β is produced and released by circulating immune effector cells known to infiltrate the esophageal epithelium in EoE, including mast cells [14] and eosinophils [15].

While TGF-β plays an established role in tissue remodeling, other profibrotic cytokines and soluble mediators can activate fibroblasts and induce ECM production [16]. IL-1β, for example, enhances the effects of TGF-β in the acquisition of the mesenchymal phenotype in human bronchial epithelial cells *in vitro* [17]. TNF-α has been implicated in the development of EMT in retinal pigment epithelial cells [18], and enhances TGF-β-induced EMT in human alveolar epithelial cells [19]. To date, the potential role for IL-1β and TNF-α in EoE-associated tissue remodeling has not been investigated.

Others have shown that cross-talk between epithelial and mesenchymal cells contributes to remodeling in other model systems [20–22]. Building upon our previous reports that human esophageal epithelial cells function as effector cells in the pathogenesis of esophageal inflammation [23,24], we hypothesized that esophageal epithelial and mesenchymal cross-talk plays a role in EoE-associated fibrosis. In this study, we show for the first time that esophageal epithelial cells prime esophageal fibroblasts to secrete fibrogenic cytokines IL-1β and TNF-α. Surprisingly, we demonstrate that these cytokines play a role in the development of EMT *in vitro*, and this can occur in a TGF-β-independent fashion. Using a primary EoE cell line grown in organotypic culture with primary fibroblasts, we further demonstrate that esophageal epithelial cells can function as innate immune effector cells in the context of EoE.

Materials and methods

Cell lines: Three human esophageal epithelial cell lines, EPC2-hTERT, EPC394, and EPC425, were grown at 37 °C in a humidified 5% CO_2 incubator, and maintained keratinocyte serum free medium (KSFM, Invitrogen, Grand Island, NY) containing human epidermal growth factor (1 ng/mL), bovine pituitary extract (50 μg/mL), and penicillin (100 units/mL) and streptomycin (100 μg/mL). The EPC2-hTERT cell line is a telomerase-immortalized and nontransformed cell line, whereas the EPC394 and EPC425 cells lines are primary cell lines obtained from an EoE (EPC394) and a non-EoE control (EPC425) patient. Fetal esophageal fibroblasts (FEF3 cells, gift of Hiroshi Nakagawa MD, PhD) and a primary fibroblast cell line (PEF429) from an adolescent patient with EoE, were maintained in Dulbecco's Minimum Essential Media (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO), and grown at 37 °C in a humidified 5% CO_2 incubator.

Primary esophageal cell lines: Esophageal biopsies were placed in Hanks BSS buffer, transferred to dispase (BD Biosciences, 50 U/mL) for 20 min at 37 °C, then trypsinized (trypsin-EDTA, GIBCO) at 37 °C. Trypsin was inactivated using soybean trypsin inhibitor (SIGMA) and biopsies were gently manually shaken. Samples were poured through a cell strainer and cells were collected in a conical tube. Cells were pelleted by centrifugation at 4 °C for 5 min. For epithelial cell isolation, pellets were resuspended in KSFM containing antibiotics and fungizone (1:500) (GIBCO). For fibroblast isolation, pellets were resuspended in DMEM with antibiotics and fungizone (1:500) (GIBCO). Cell suspensions were then seeded in tissue culture plates. Cells were used at passage 2–3.

Conditioned epithelial media (*CEM*) *stimulation*: Conditioned epithelial media (CEM) was collected from confluent EPC2-hTERT cells grown in complete KSFM, and used to stimulate fibroblast monolayers for 3 and 6 h. Prior to stimulation of fibroblasts, CEM was supplemented with 10% FBS. Fig. 1A shows the schematic of the experimental design. For control conditions,

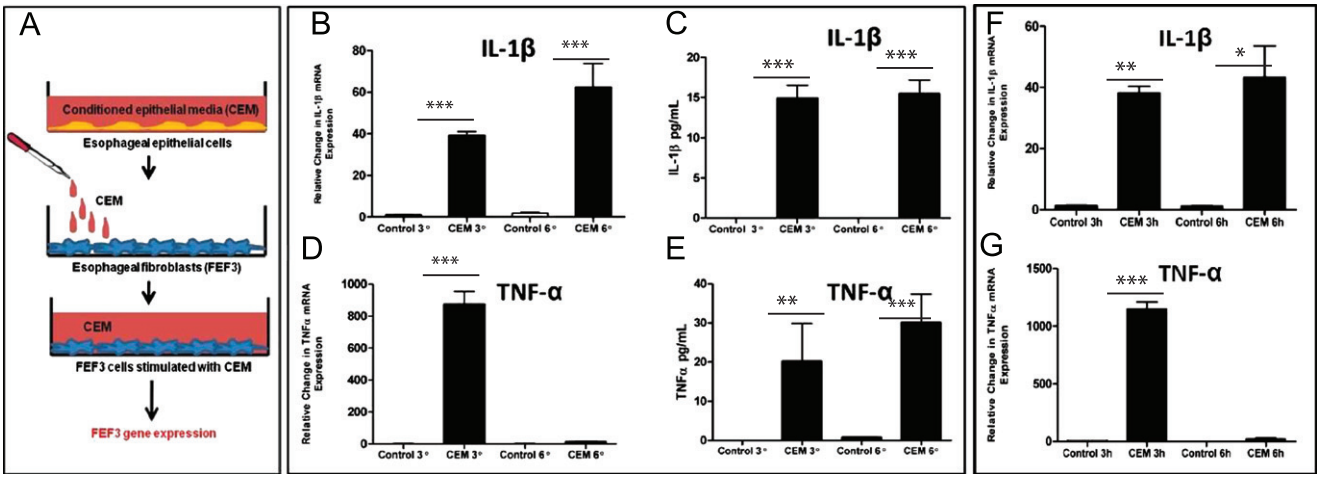

Fig. 1 – Conditioned epithelial media (CEM) primes fibroblasts to secrete proinflammatory cytokines. (A) Schematic of experimental design: conditioned epithelial media (CEM) from esophageal epithelial cells (EPC2-hTERT) were transferred to fetal esophageal fibroblasts (FEF3 cells). Media was collected at various time points for quantification of FEF3-secreted cytokines, and FEF3 cells were harvested for analysis of gene expression. (B) and (D) mRNA expression of IL-1β and TNFα by FEF3 cells at various time points after CEM stimulation. (C) and (E) Quantification of IL-1β and TNFα secretion by FEF3 cells at various time points following CEM stimulation. (F) and (G) mRNA expression of IL-1β and TNFα expression by primary esophageal fibroblasts (PEF429) from an adolescent EoE patient following stimulation with CEM for various time points. Data shown are representative of at least 3 individual experiments. Error bars represent standard error. *$p<0.05$, **$p<0.01$, ***$p<0.001$, NS=not significant.

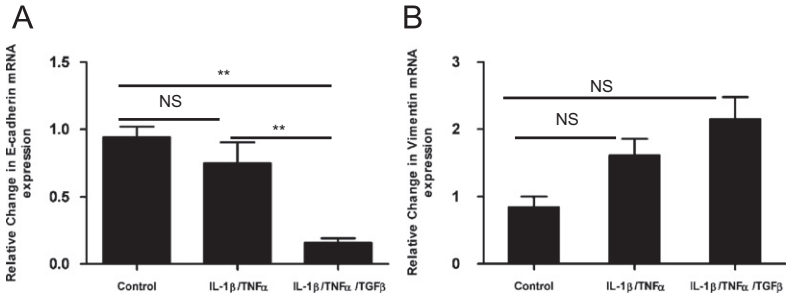

Fig. 2 – Stimulation of esophageal epithelial cell stimulation with IL-1β and TNF-α induces expression of mesenchymal genes and suppresses expression of epithelial markers, features of EMT which are further enhanced by TGF-β. (A) mRNA expression of epithelial-specific E-cadherin by EPC2-hTERT cells after 3 weeks of stimulation with combinations of IL-1β, TNFα, and TGF-β. (B) Expression of mesenchymal marker vimentin by EPC2-hTERT cells after 3 weeks of dual cytokine (IL-1β/TNFα) or triple cytokine (IL-1β/TNFα/TGF-β) stimulation. Data shown are representative of at least 3 individual experiments. *p*-values were calculated based upon comparisons to unstimulated conditions. *$p<0.05$, **$p<0.01$, NS=not significant.

unconditioned complete KSFM was supplemented with 10% FBS. After stimulation with CEM, media was collected for ELISA, and fibroblasts were harvested for RNA isolation.

Stimulation of epithelial cells with recombinant cytokines: EPC2-hTERT cells were seeded in 6 well plates at a density of 3×10^5 cells/well 1 day prior to stimulation. Cells were stimulated in triplicate with combinations of human recombinant TGF-β(R&D Systems, Minneapolis, MN) (10 ng/mL), IL1-β (Sigma, Saint Louis, MO) (10 ng/mL), and TNF-α (R&D) (40 ng/mL). Media, including cytokines, was refreshed weekly, and cells were harvested after 3 weeks of stimulation for RNA isolation.

Fibroblast conditioned media (FCM) stimulation: FEF3 cells were first stimulated with CEM. After 6 h, this fibroblast-conditioned media (FCM) was then used to stimulate fresh monolayers of EPC2-hTERT cells for 3 weeks. A schematic of the experimental design is shown in Fig. 3A. For control conditions, EPC2-hTERT cells were treated for 3 weeks with unconditioned KSFM (+10% FBS) which had been applied to FEF3 cells for the same time points. For inhibition studies, cells treated with FCM were also co-treated with infliximab (Remicade) (1 μg/mL, gift of Monica Darby), anti-IL1-R (Anakinra) (40 ng/mL, R&D), or both. FCM, KSFM, or the inhibitors were refreshed weekly until day 21, when EPC2-hTERT cells were either harvested for RNA or used for immunofluorescence.

RNA isolation and quantitative RT-PCR. RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. RNA samples were reverse transcribed using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). Preformulated TaqMan Gene Expression Assays were purchased from Applied Biosystems for human TNF-α, IL-1 β, TGF-β, vimentin, E-cadherin, αSMA, and GAPDH. Quantitative RT-PCR was performed by using Taqman Fast

Universal PCR Master Mix kitand reactions were performed in triplicate using 96 well optical plates on a StepOnePlus Real-Time PCR System (Applied Biosystems). GAPDH was used as an endogenous control to normalize the samples using C_T method of relative quantification, where C_T is the threshold cycle.

Enzyme-linked immunosorbent assay (ELISA): TNF-α, IL1-β, and TGF-β were quantified in culture supernatants using ELISA (R&D), using manufacturer's recommendations.

Immunofluorescence: For cell monolayers, esophageal epithelial cells were seeded in glass chamber slides at a density of 3×10^5 cells/chamber . Following 3 weeks of FCM stimulation, cells were fixed and permeabilized using methanol/acetone at −20 °C for 10 min, followed by incubation in primary antibody [(mouse anti-human E-cadherin (BD Bioscience) (1:200), mouse anti-human αSMA (Sigma) (1:1000)] for 2 h at 4 °C. Secondary antibody [rabbit anti-mouse Dylight (Jackson Immunoresearch Laboratories) (1:600)] was applied for 1 h at room temperature. Slides, mounted with DAPI mounting media (VECTAshield), were viewed using an Olympus BX51 microscope.

For organotypic culture and patient biopsy slides, sections were re-hydrated and boiled in sodium citrate buffer, then incubated with the primary antibodies [chicken anti-human vimentin (Novus Biologicals, Littleton, CO) 1:5000, E-cadherin 1:200, αSMA 1:1000] at 4 °C, followed by secondary anti-chicken antibody (Jackson Immunolaboratories) (1:600) or anti-mouse Dylight antibody (1:600) for 1 h at room temperature prior to mounting in DAPI mounting media.

Organotypic cell culture (*OTC*): OTC models were constructed using previously published methods [25]. Briefly, 5×10^5 esophageal epithelial cells (EPC2-hTERT, primary EoE cell line EPC394, primary non-EoE cell line EPC425) were seeded onto a collagen matrix, containing 7.5×10^4 fetal esophageal fibroblast

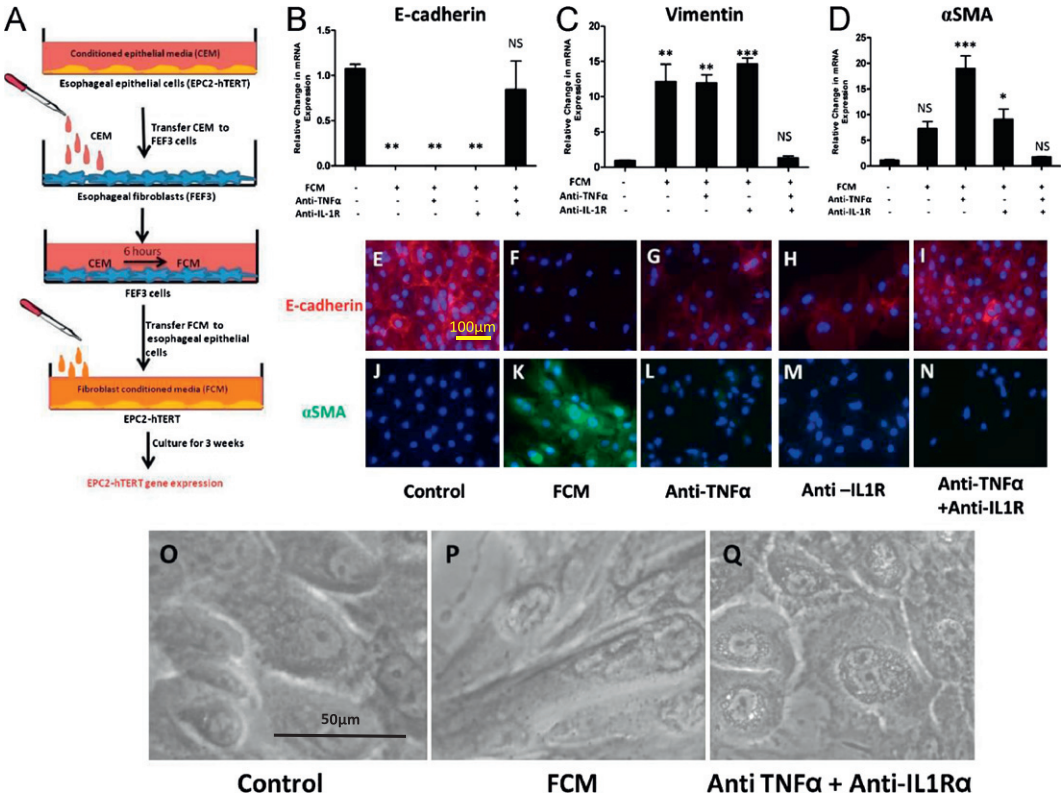

Fig. 3 – Stimulation of esophageal epithelial cells with fibroblast conditioned media (FCM) leads to features of EMT, in an IL-1β and TNFα-dependent fashion. (A) Schematic of experimental design: Following stimulation of FEF3 cells with CEM (2 days), this "fibroblast conditioned media" (FCM) was harvested and transferred to fresh EPC2-hTERT cells. After 3 weeks of FCM stimulation in the presence of absence of competitive inhibitors of IL-1β and/or TNFα, EPC2-hTERT cells were harvested for mRNA isolation, or immunolocalization of mesenchymal/epithelial markers. (B)–(D) mRNA expression of E-cadherin, vimentin, and αSMA by EPC2-hTERT cells after stimulation with FCM in the presence or absence of anti-TNFα mAb (Remicade) and/or anti-IL-1R (Anakinra). (E) and (J) Constitutive expression of epithelial E-cadherin (red) and αSMA (green) by EPC2-hTERT cells. Nuclei are counterstained with DAPI (blue). (F) and (K) Loss of E-cadherin expression (red) and enhanced αSMA (green) expression by EPC2-hTERT cells following 3 weeks of stimulation with FCM. (G) and (H) Partial recovery of E-cadherin expression by EPC2-hTERT cells stimulated with FCM and anti-TNFα mAb (Remicade) or anti-IL-1R (Anakinra). (L) and (M) Absence of αSMA in EPC2-hTERT cells treated with FCM in the presence anti-TNFα mAb (Remicade) or anti-IL-1R (Anakinra). (I) and (N) Combination of anti-TNFα mAb (Remicade) and anti-IL-1R (Anakinra) protects EPC2-hTERT cells from effects of FCM stimulation. (O) and (P) Morphology of EPC2-hTERT cells before and after FCM stimulation. (Q) Effect of anti-TNFα mAb and anti-IL-1R upon EPC2-hTERT morphology. *p*-values were calculated based upon comparisons to unstimulated conditions. $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, NS=not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1 – Clinical characteristics of subjects 394 and 425, which were used to generate primary esophageal epithelial cell lines EPC394 and EPC425. Esophageal biopsies from subject 429 were used to generate the primary esophageal fibroblast cell line PEF429.

Subject	394	425	429
Age (years)	11	12	13
Gender	Male	Male	Female
Symptoms	Dysphagia	Heartburn, abdominal pain	Dysphagia
Medications	Lansoprazole, allergy shots	Omeprazole, cetirizine	Omeprazole
Eosinophils per hpf	37	0	15
Diagnosis	EoE	Abdominal pain	EoE
Esophageal cell line	Epithelial (EPC 394)	Epithelial (EPC 425)	Fibroblast (PEF429)

cells (FEF3). On the fourth day after seeding, epithelial cells were raised to the air-liquid interface and cultured for another 6 days. Cultures were harvested and fixed with 10% neutral buffered formalin and embedded in paraffin. Sections were used for immunofluorescence.

Trichrome staining: Human biopsy slides were deparaffinized, rehydrated, and stained using a Masson trichrome staining protocol [26].

Human subjects: The human subjects protocol was approved by the Institutional Review Board at the Children's Hospital of Philadelphia. Following informed consent, additional esophageal pinch biopsies were obtained during routine diagnostic esophagogastroduodenoscopy (EGD) for isolation of primary esophageal epithelial or fibroblast cell lines. Consistent with recently published clinical guidelines, the diagnosis of EoE was made histologically by the presence of 15 or more esophageal epithelial eosinophils per high powered field (hpf), hyperplasia of the basal epithelium, and the absence of tissue eosinophilia in the more distal GI tract [27]. All subjects were on high dose PPI therapy for at least 8 weeks prior to biopsy.

Statistical analysis: A two-tailed Student's *t*-test was used for analysis of Fig. 1, and a one-way ANOVA and post-hoc comparison with Bonferroni was used to analyze Figs. 2 and 3. A p value of ≤ 0.05 was considered to be statistically significant.

Results

1) Conditioned esophageal epithelial media primes esophageal fibroblasts to secrete IL-1β, TNF-α, but not TGF-β.As a first step in investigating esophageal epithelial and mesenchymal cross-talk, we determined whether esophageal fibroblasts could sense factors released by esophageal epithelial cells *in vitro*. We investigated cross-talk using the immortalized nontransformed EPC2-hTERT esophageal epithelial cells, and the primary fetal esophageal fibroblast (FEF3) cell line. EPC2-hTERT cells exhibit a normal karyotype, do not undergo a slow-growth phase, and have been routinely used through 200 passage days (PD) by others [28]. EPC2-hTERT cells in this study were used between 30 and 50 PD. CEM from confluent EPC2-hTERT cells was used to stimulate confluent FEF3 cells for various time points. Unconditioned, fresh KSFM was used for control conditions. A schematic of the experimental design is shown in Fig. 1A. To analyze the pro-fibrotic response, we quantified FEF3 mRNA expression and secretion of IL-1β, TNF-α, and TGF-β in response to CEM stimulation. Fibroblast mRNA expression of IL-1β peaked at the 6 h time point following stimulation, with corresponding protein secretion sustained at both the 3 and 6 h time points (Fig. 1B and C). Robust mRNA expression of TNF-α was detected at 3 h following CEM stimulation, with protein secretion sustained at 3 and 6 h post-stimulation (Fig. 1D and E). Protein concentrations of TNF-α and IL-1β remained unchanged through 5 days (data not shown). Notably, there was no detectable TGF-β in the CEM, nor did CEM induce any mRNA expression or protein secretion of TGF-β from stimulated FEF3 cells (data not shown).
Fetal-derived fibroblasts, including FEF3 cells, may have distinct functional differences from mature fibroblasts [29–31]To control for this possibility, CEM was also used to stimulate primary esophageal fibroblasts isolated from an adolescent patient with EoE (PEF429), and CEM-induced mRNA expression of IL-1β, TNF-α, and TGF-β were quantified. The clinical characteristics of the EoE subject from which the esophageal fibroblasts were acquired are shown in Table 1. PEF429 response to CEM paralleled that of FEF3 cells, with significant induction in IL-1β (Fig. 1F) and TNF-α (Fig. 1G), but not TGF-β (not shown). Based upon the similarities in response to CEM between the two fibroblast cell lines, the remainder of experiments in this study were performed using the FEF3 cell line.
2) Exposure of esophageal epithelial cells to pro-fibrogenic cytokinesIL-1β, TNF-α, and TGF-β leads to features of EMT *in vitro*. We hypothesized that CEM-induced production of fibroblast-derived cytokines IL-1β and TNF-α might exert pro-fibrogenic effects upon esophageal epithelial cells. To recapitulate this hypothesized paracrine signaling pathway *in vitro*, we cultured EPC2-hTERT cells in the presence of recombinant human IL-1β and TNF-α, and quantified mRNA expression of the epithelial-specific marker E-cadherin and the mesenchymal marker vimentin after 3 weeks in culture. The 3 week time point was chosen based upon the findings of Ohashi et al. who previously demonstrated that EPC2-hTERT cells undergo maximal TGF-β-induced transition to spindle-like morphology after 21 days of continuous cytokine exposure *in vitro* [10]. Although fibroblast expression and secretion of TGF-β was not detected in our model system, we hypothesized that exogenous TGF-β might further enhance the pro-fibrogenic effects of IL-1β and TNFα upon EPC2-hTERT cells.
Though not statistically significant, the expression of the epithelial marker E-cadherin was modestly reduced by IL-1β/TNF-α stimulation. This effect was enhanced by the addition of TGF-β, which led to a significant reduction in E-cadherin expression (Fig. 2A). Notably, 3 week stimulation with TGF-β alone led to a reduction in E-cadherin expression, though not statistically significant (data not shown). Though

the effects of these pro-fibrotic cytokines upon vimentin expression did not reach statistical significance, expression of this mesenchymal marker trended upward following IL-1β/TNF-α stimulation, and was further increased following the addition of exogenous TGF-β, suggestive of EMT (Fig. 2B).

3) Esophageal epithelial exposure to fibroblast conditioned media (FCM) leads to features of EMT in a TNF-α and IL-1β -dependent fashion. To further interrogate epithelial-fibroblast cross-talk, we stimulated EPC2-hTERT cells with media harvested from CEM-stimulated FEF3 cells, which was designated as "fibroblast conditioned media" (FCM). Unconditioned cell culture media not previously in contact with epithelial cells, was applied to FEF3 cells for control conditions. A schematic of the experimental design is shown in Fig. 3A. To determine the role of IL-1β and TNF-α in the development of FCM-induced EMT, competitive inhibition experiments were performed in the presence of combinations of anti-TNF-α (Remicade) and anti-IL-1R (Anakinra) [32,33]. Competitive inhibition of TGF-β signaling was not performed based upon the absence of TGF-β mRNA expression in FCM-stimulated esophageal epithelial cells (data not shown). Following 3 weeks of culture in FCM, the mRNA expression of E-cadherin was significantly reduced (Fig. 3B). This effect was not reversed with competitive inhibition using either anti-TNF-α or anti-IL-1R alone. Remarkably, however, combined anti-TNF-α and anti-IL-1R almost completely rescued epithelial cells from the FCM-induced suppression of E-cadherin. This pattern of expression was also evident using immunofluorescent staining for E-cadherin (Fig. 3E–I).

Consistent with EMT, FCM stimulation also enhanced the expression of mesenchymal genes. FCM significantly induced the mRNA expression of vimentin, an effect which was reversible through co-inhibition of TNF-α and IL-1 signaling. Similar to E-cadherin, the effect of FCM upon vimentin expression was not affected by either inhibitor alone (Fig. 3C). Though not statistically significant, the effect of FCM stimulation upon αSMA mirrored that of vimentin (Fig. 3D). Immunostaining for αSMA demonstrated enhanced expression of this mesenchymal marker in FCM-stimulated cells, which appeared to be rescued by anti-TNF-α and anti-IL-1R, both alone and in combination (Fig. 3J–N). Unexpectedly, although anti-TNF-α enhanced the mRNA expression of αSMA, this was not reflected in immunofluorescent staining for αSMA (Fig. 3L).

Cell morphology was also altered during the 3 weeks of FCM exposure. In contrast to control cells, FCM-stimulated epithelial cells developed elongated, spindle-like morphology. Competitive inhibition of TNF-α and IL-1R rescued epithelial cells from these morphologic changes (Fig. 3O–Q).

4) Primary EoE esophageal epithelial cells exhibit features of EMT when grown in organotypic cell culture. To explore epithelial and mesenchymal cross-talk within physiologic context, we used primary esophageal epithelial cell lines grown in organotypic cell culture (OTC) models. In the organotypic model, esophageal epithelial cells and fibroblasts grow in physiologic context, where direct cell–cell contact is maintained for over 2 weeks. We hypothesized that OTC-cultured primary esophageal epithelial cells derived from an EoE subject (EPC394) would exhibit

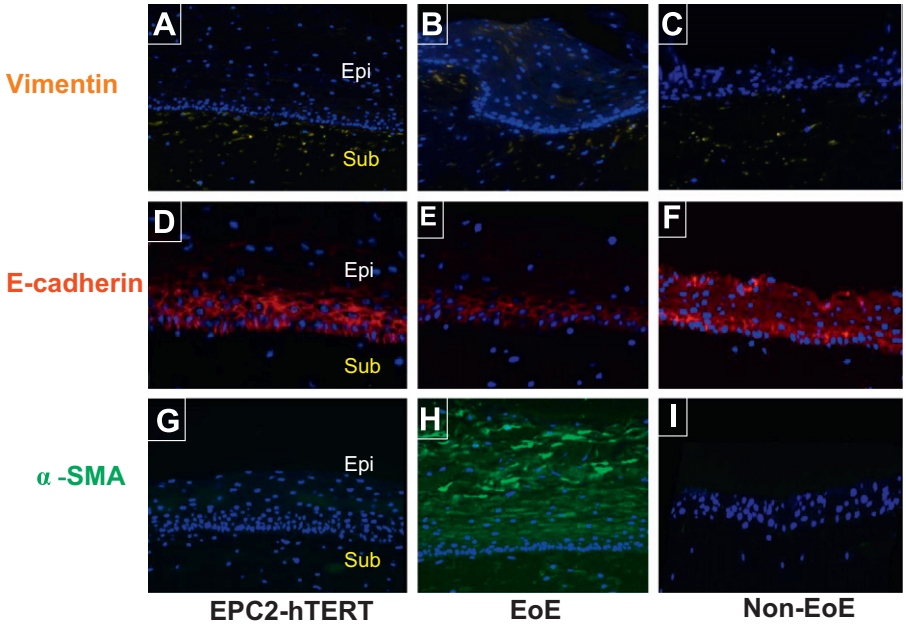

Fig. 4 – Primary esophageal epithelial cells from an EoE subject exhibit fibrogenic behavior compared to non-EoE control when grown in organotypic cell culture (OTC). Primary esophageal epithelial cells (passage 3) were harvested from subjects 394 (EoE) and 425 (non-EoE), and seeded onto a matrix of FEF3 cells within a collagen matrix. OTC was also constructed using EPC2-hTERT cells. Following epithelial differentiation and stratification, OTC were harvested for immunolocalization of EMT markers. (A)–(C) Expression of mesenchymal marker vimentin (yellow) in EPC2-hTERT, EPC394 (EoE) and EPC425 (non-EoE) OTC. (D)–(F) E-cadherin expression (red) in EPC2-hTERT, EPC394 (EoE) and EPC425 (non-EoE) grown in OTC. (G)–(I) Expression of α-SMA (green) in OTC constructed using EPC2-hTERT, EPC394, and EPC425 cell lines. In all sections, nuclei are counterstained with DAPI (blue). Epithelial (Epi) and subepithelial (Sub) compartments are labeled. Images shown are at 200× magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

enhanced features of EMT, compared to both a non-EoE subject (EPC425) and the EPC2-hTERT cell line. The clinical characteristics of the EoE and control subject are outlined in Table 1.

Using previously published methods, three OTC models were constructed using esophageal epithelial cells (EPC2-hTERT, EoE-EPC394, and non-EoE-EPC425) seeded on FEF3 cells embedded in a collagen matrix. Following differentiation and stratification, cultures were harvested for immunolocalization of E-cadherin, αSMA, and vimentin. Consistent with our findings in cell monolayers, we observed a modest decrease in E-cadherin expression in the EoE cell line, along with increased expression of mesenchymal markers αSMA and vimentin. In contrast, the non-EoE control EPC425 cell line exhibited similar expression of both epithelial and mesenchymal markers compared to the EPC2-hTERT cell line (Fig. 4).

5) Validation of fibrosis and EMT *in vivo* in EoE. In order to validate our *in vitro* organotypic findings, we evaluated the esophageal biopsy samples from the EoE and non EoE subjects from which the EoE and non-EoE primary epithelial cell lines were derived. Trichrome staining revealed that the EoE subject not only exhibited densely packed collagen within the subepithelial compartment, but also had extension of collagen deposition into the papillae (Fig. 5A). In contrast, loose collagen fibrils were seen in the subepithelial compartment of the non-EoE subject (Fig. 5E). Similar to the findings described by Kagalwalla et al. [9] markers of EMT were detected using immunofluorescence in biopsies from the EoE subject (decreased E-cadherin, increased αSMA and vimentin, Fig. 5B–D) compared to the non-EoE subject (Fig. 5F–H).

Discussion

In this study, we show for the first time that cross-talk between esophageal epithelial cells and esophageal fibroblasts leads to features of EMT *in vitro*. We demonstrate that two cytokines previously implicated in other models of cross-talk and fibrosis, IL-1β and TNF-α, may play an inciting role in the development of EMT. Our results support the recent report by Kagalwalla et al, which demonstrated that EMT occurs in the esophageal epithelium of EoE subjects [9]. Importantly, however, we now demonstrate that some of the cardinal features of EMT, acquisition of mesenchymal markers and loss of epithelial markers, can occur in a TGF-β independent fashion. Our *in vitro* organotypic model further corroborates our hypothesis of epithelial-mesenchymal cross-talk, and demonstrates that some features of EMT can occur in the absence of immune cells, tissue injury, or chronic inflammation.

In EoE, TGF-β has been suggested as a primary effector of fibrosis, supported by immunostaining for TGF-β [9] and its signaling molecule phospho-Smad 2/3 [8] in esophageal biopsies of EoE patients. Previous reports demonstrate that granulocyte populations which infiltrate the esophageal mucosa, including mast cells [34]and eosinophils [15] secrete TGF-β, further supporting the notion that this cytokine may play a role in EoE fibrogenesis. The role of TGF-β in myofibroblast development [7,13] and fibrogenesis [2,11,35] has been very well-characterized in other model systems. Interestingly, eosinophils, when co-cultured with fibroblasts, have also been shown to activate fibroblasts by releasing both TGF-β and IL1-β [36].

To our knowledge, this is the first study which looks beyond eosinophil and mast cell-derived cytokines as the major driving force behind tissue remodeling in EoE. In our granulocyte-free model, TGF-β is not secreted by the epithelium or by epithelial-primed fibroblasts. Our results contrast with the findings of others who have previously shown that epithelial-derived TGF-β contributes to the development of EMT via autocrine signaling [37,38]. In the absence of the inflammatory triggers and granulocytes important to EoE pathogenesis, the possibility that autocrine TGF-β signaling contributes to EoE-associated tissue remodeling cannot be excluded (Fig. 6).

Notably, the effect of IL-1β and TNF-α stimulation upon the development of EMT in EPC2-hTERT cells was enhanced by the

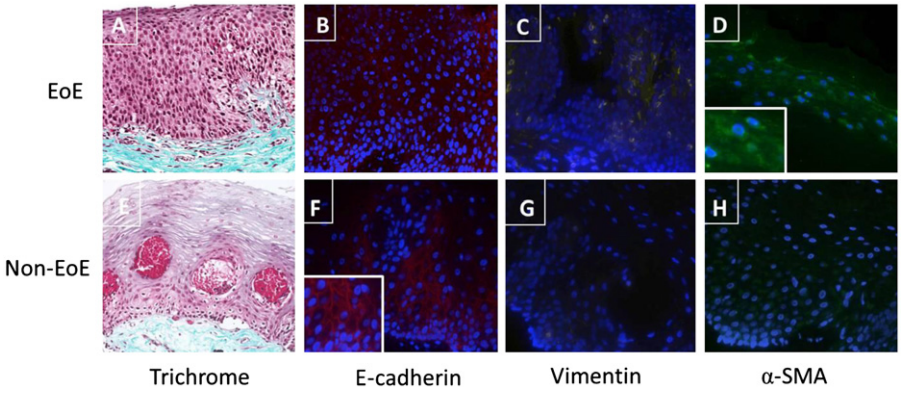

Fig. 5 – Expression of subepithelial collagen and mesenchymal/epithelial markers in native biopsies from EoE and control subjects used for primary esophageal epithelial cell lines. (A) and (E) Trichrome stain of esophageal biopsy from subjects 394 and 425 shows differential subepithelial collagen deposition (blue) in EoE (394) and non EoE (425) subjects. (B) and (F) Reduced epithelial E-cadherin (red) expression in EoE subject compared to normal control. (F) Inset shows magnified detail of E-cadherin in the normal control. (C) and (D) Expression of mesenchymal marker vimentin (yellow) and α-SMA (green) in EoE subject. (D) Inset shows magnified detail of α-SMA expression in the EoE biopsy sample. (G) and (H) Vimentin and α-SMA expression in control subject biopsy. Images are shown at 200× magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

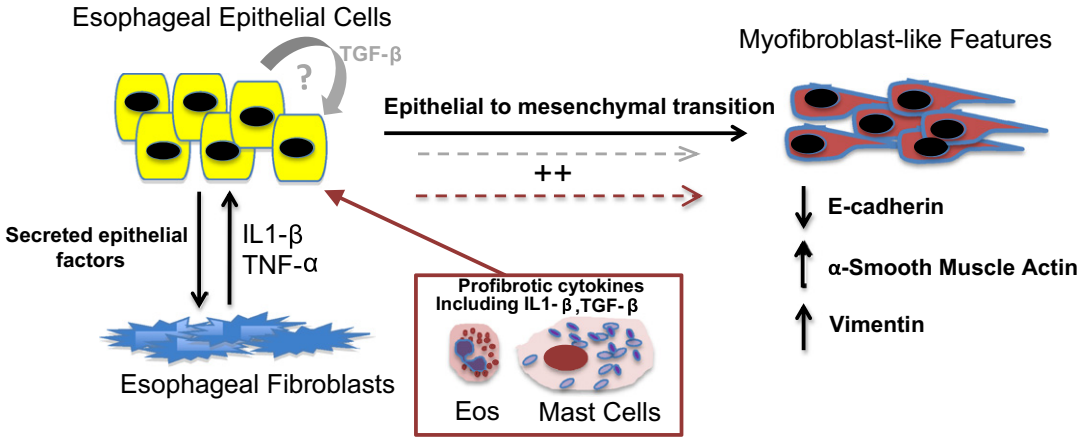

Fig. 6 – Proposed mechanism of esophageal epithelial-mesenchymal cross talk and EMT. Together, our model suggests that in a genetically predisposed host, factors secreted by esophageal epithelial cells prime esophageal fibroblasts to secrete cytokines including IL-1β and TNF-α. Fibroblast-derived cytokines IL-1β and TNF-α, then stimulate adjacent esophageal epithelial cells to lose epithelial markers (E-cadherin) and gain expression of mesenchymal markers including vimentin and α-SMA. Though our *in vitro* model suggests that while these features of EMT can occur in the absence of TGF-β, granulocyte-derived TGF-β and IL-1β (maroon box and arrows) and potentially autocrine signaling by epithelial-derived TGF-β (gray arrows), may further enhance the development of EMT *in vivo*.

addition of TGF-β through suppression of epithelial E-cadherin and induction of mesenchymal vimentin expression. This supports previous findings that TGF-β alone is in sufficient to induce fibrosis in specific model systems. In a murine model of systemic sclerosis, Mori et al. found that skin fibrosis was induced only when mice were injected with both connective tissue growth factor (CTGF) and TGF-β [39]. Fattouh et al. found that allergic airway remodeling can occur independently from TGF-β and may depend on IL-13 and other eosinophil derived factors [40]. Overall, our findings may suggest a pathway by which, in a genetically predisposed individual, esophageal epithelial and mesenchymal cross talk participates in the pathogenesis of EMT. The addition of environmental triggers including diet [41] and pollen [42,43], may lead to the infiltration and activation of innate granulocyte populations (eosinophils, mast cells) which secrete TGF-β and IL1β [36], synergistically enhancing tissue remodeling in EoE (Fig. 6).

Unexpectedly, FCM was a more potent inducer of EMT (Fig. 3) compared to the combined effect of recombinant cytokines (Fig. 2). Although our reductionist approach suggests an important and novel role for IL-1β and TNF-α in FCM-induced EMT, this observation clearly suggests that other soluble mediators play a role in our cell culture model of EMT. Some candidates for future studies include growth factors (including insulin-like growth factor I, epidermal growth factor, basic fibroblast growth factor, platelet-derived growth factor) and cytokines (IL-1, IL-4, IL-6, IL-13, and IL-21) known to activate fibroblasts in other models [16,44]. Alternatively, the role of Notch signaling in epithelial-mesenchymal cross talk may be explored, as Notch signaling has been previously shown to play a role in EMT in both TGF-β-dependent [45,46] and TGF-β independent [47] model systems.

The role of IL-1β and TNF-α as profibrotic mediators has been reported in other models. IL-1β has been implicated in pancreatic fibrosis and liver fibrosis. Shen et al. showed that intraperitoneal injections of anti-IL1-R in mice with chronic pancreatitis attenuated pancreatic fibrosis [48]. Furthermore, IL-1β−/− mice fed a high-fat diet are protected against steatohepatitis and liver fibrosis compared to wild type controls [49]. Likewise, TNF-α has been implicated in skin EMT [50]. Interestingly many studies suggest that both of these cytokines require TGF-β as a co-stimulant in order to induce fibrosis [51,52].

Although the use of the FEF3 fetal fibroblast cell line has been validated in organotypic models of esophageal cancer [53,54], a potential weakness of our study is the exclusive use this fetal cell line. Indeed, it has been proposed that fetal and adult fibroblasts have differential migratory abilities [29] and different responses to TGF-β [31]. In addition, variations in fibroblast phenotype and activation state are known to influence the invasive behavior of the adjacent epithelium in cell culture models of esophageal cancer [55]. However, functional comparisons of esophageal fibroblasts in pediatric EoE have not been reported. Though our results show that primary esophageal fibroblasts from a single EoE patient have similar innate immune responsiveness to CEM compared to the fetal FEF3 cell line (Fig. 1F and G) future studies will investigate interactions between esophageal epithelial cells from EoE subjects with esophageal fibroblasts from age-matched controls.

Kagalwalla's study showed that treatment of EoE with dietary restriction or topical corticosteroids (TC) reduced tissue eosinophil load and EMT scores, suggesting that therapies which reduce eosinophil counts ameliorate EoE by reversing EMT. While the precise mechanisms by which dietary restriction and TC improve EoE esophageal inflammation are unknown, it is likely that their anti-inflammatory effects involve esophageal epithelial immune responses. In asthma, the effects of budesonide upon bronchial epithelial cells have been well-described [56–58], and the efficacy of topical corticosteroid therapy in EoE further supports a role for esophageal epithelial cells in EoE pathogenesis. Interestingly, Mulder et al. showed that esophageal epithelial cells can internalize, process, and present ovalbumin to activated T-cells, implicating esophageal epithelial cells as nonprofessional antigen presenting cells in diet-triggered EoE [59]. In the current study, our results now suggest an additional role for esophageal epithelial

cells as profibrogenic effector cells in EoE fibrosis. Continued studies using additional primary esophageal epithelial and fibroblast cell lines will be important to further elucidate signaling mechanisms involved in pathogenesis of this complex disease.

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Conflicts of interest statement

None.

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Basophils and allergic inflammation

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Basophils were discovered by Paul Ehrlich in 1879 and represent the least abundant granulocyte population in mammals. The relative rarity of basophils and their phenotypic similarities with mast cells resulted in this cell lineage being historically overlooked, both clinically and experimentally. However, recent studies in human subjects and murine systems have shown that basophils perform nonredundant effector functions and significantly contribute to the development and progression of T_H2 cytokine–mediated inflammation. Although the potential functions of murine and human basophils have provoked some controversy, recent genetic approaches indicate that basophils can migrate into lymphoid tissues and, in some circumstances, cooperate with other immune cells to promote optimal T_H2 cytokine responses *in vivo*. This article provides a brief historical perspective on basophil-related research and discusses recent studies that have identified previously unappreciated molecules and pathways that regulate basophil development, activation, and function in the context of allergic inflammation. Furthermore, we highlight the unique effector functions of basophils and discuss their contributions to the development and pathogenesis of allergic inflammation in human disease. Finally, we discuss the therapeutic potential of targeting basophils in preventing or alleviating the development and progression of allergic inflammation. (J Allergy Clin Immunol 2013;132:789-801.)

Abbreviations used

AD:	Atopic dermatitis
APC:	Antigen-presenting cell
AR:	Allergic rhinitis
BaP:	Basophil precursor
BAT:	Basophil activation test
CIU:	Chronic idiopathic urticaria
EMH:	Extramedullary hematopoiesis
EoE:	Eosinophilic esophagitis
FDA:	US Food and Drug Administration
GMP:	Granulocyte-monocyte progenitor
HDM:	House dust mite
HSC:	Hematopoietic stem cell
LTC_4:	Leukotriene C_4
MCP:	Mast cell precursor
MyD88:	Myeloid differentiation primary response gene (88)
TSLP:	Thymic stromal lymphopoietin

Key words: *Basophil, allergy, T_H2 cytokine, thymic stromal lymphopoietin, allergic rhinitis, asthma, atopic dermatitis, urticaria, food allergy, eosinophilic esophagitis, IgE*

Despite being the least frequent granulocyte population in the mammalian body, the accumulation of basophils has been reported in a number of human disease states, including allergic disease, organ rejection, autoimmunity, and cancer. For example, basophils are thought to contribute to the pathogenesis of allergic contact dermatitis,[1] atopic dermatitis (AD),[2] allergic drug reactions,[3] immediate hypersensitivity reactions (eg, anaphylaxis),[3] asthma,[4-6] bullous pemphigoid,[2] lupus nephritis,[7] Crohn disease,[3] skin and kidney allograft responses,[8,9] and acute and chronic myelogenous leukemia (Fig 1).[10,11] Furthermore, the ***basophil activation test*** (BAT) has been used experimentally to detect allergic reactions to drugs, food, and venom in patients.[12,13] Although basophil responses are associated with a number of diseases, the focus of this review will be to examine the current understanding of the function of basophils within the context of allergic inflammation.

The development of new murine genetic tools and models of inflammation, coupled with the development of more selective reagents to detect and manipulate basophils, has resulted in novel insights into the potential contribution of basophils to human disease. In this review we provide a brief historical perspective on basophil-related research. Next, we focus on the current understanding of the role basophils play in promoting T_H2 cytokine–mediated inflammation and allergic disease. We discuss how heterogeneity in basophil responses might contribute to the

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Terms in boldface and italics are defined in the glossary on page 790.

complexity of allergic disease states and how a better understanding of basophil biology might lead to the development of new therapeutic strategies to alleviate allergic inflammation.

BASOPHILS: A HISTORICAL PERSPECTIVE

The German physician-scientist Paul Ehrlich identified basophils in 1879 based on their unique microscopic appearance after being exposed to basic stains.[14] Basophils are the least abundant granulocyte population in the peripheral blood, comprising less than 1% of all leukocytes.[15,16] This fact made early research on basophils difficult and promoted the notion that their lack of abundance equated to a lack of biological importance. Subsequent studies that occurred almost a century later determined that basophils contain histamine and express the high-affinity IgE receptor FcεRI.[16-18] However, because of their relative lack of abundance and phenotypic and functional similarities to mast cells, basophils were regarded as a redundant granulocyte population lacking unique functions. In addition, the presence of basophils in the peripheral blood allowed them to be more easily obtained than tissue-resident mast cells, and as a result, basophils began to be used as surrogates for mast cells in functional assays to better understand granulocyte biology.[16] However, subsequent studies directly comparing mast cell and basophil populations began to elucidate that basophils exhibit unique developmental, phenotypic, and functional features (see below).[19-23] Several seminal studies in the 1970s and 1980s using both rats and guinea pigs demonstrated that basophil populations expand dramatically in response to various helminth parasites and parasite-derived antigens, suggesting that basophils might play a role in protective T_H2 cytokine–mediated immunity to some parasites.[24-28] Despite these observations, the unique contributions of basophils to the development of allergic inflammation could not be studied at the time because of the lack of mouse models and tools to selectively manipulate basophil responses *in vivo*.

In 1981, a histamine-containing cell population termed the "persisting cell" or P cell was identified in mice and was the first identification of a basophil-like cell population.[29] Subsequent studies the following year by Dvorak et al[30] elaborated on these original observations and identified a granular cell population in the bone marrow of mice that resembled basophils in rats. This study was the first to officially report the identification of basophil populations in mice. The identification of basophils in mice enabled technologic advancements to directly test the pathways that regulate their development and contribution to immunity, inflammation, and disease.

Since the identification of murine basophils in 1982, significant advances in basophil biology have been made. For example, the development of 2 mouse models by the laboratories of Paul and colleagues[31] and Locksley and colleagues[32] that expressed green fluorescent protein under the control of the IL-4 promoter allowed for a series of studies that significantly enhanced our understanding of basophil biology. These murine models facilitated the discovery that mature eosinophils, mast cells, and basophils constitutively express IL-4/expressed green fluorescent protein

GLOSSARY

BASOPHIL ACTIVATION TEST: The basophil activation test initially measured the release of histamine from activated basophils. However, the current technique uses flow cytometry to measure basophils that express activation markers, including CD63, CD203c, or both.

CD200R: CD200R has been shown to be expressed on both human and murine basophils and mast cells. The anti-CD200R antibody (Ba103) has been employed to deplete murine basophils *in vivo*.

CD203: CD203 (also known as CD203c) is a transmembrane ectoenzyme known as ectonucleotide pyrophosphatase phosphodiesterase 3 (NPP3) that marks activated basophils. CD203 can be found after treatment with IL-3, is upregulated after treatment with IgE or allergen, and is present on basophils in allergic subjects. Both CD203 and CD63 can be used to gauge basophil activation.

C/EBPα: C/EBP is a family of transcription factors known as CCAAT-enhancer binding proteins. The protein family structure contains a basic region and a leucine zipper motif for dimerization and DNA binding. C/EBPα expression is necessary for the maturation and development of basophils from progenitor cell populations.

DIPHTHERIA TOXIN RECEPTOR: Diphtheria toxin can be used experimentally to deplete specific cell types in transgenic mice expressing the high-affinity simian diphtheria toxin receptor under the control of a cell-specific promoter. This technique has been used to deplete murine mast cells and basophils *in vivo*.

GATA-2: GATA transcription factors form a family of zinc-finger DNA-binding proteins. GATA-1, GATA-2, and GATA-3 are expressed in the hematopoietic system, and GATA-3 is essential for the expression of T_H2 cell–associated cytokines, such as IL-4, IL-5, and IL-13. GATA-2 expression is necessary for the development of basophil populations from hematopoietic stem cells.

GERM-FREE MICE: Mice that are born and raised under aseptic conditions are referred to as gnotobiotic, a term that includes germ-free mice. Germ-free mice can then be inoculated with only certain species of microbial flora to understand the host-microbiome interaction. Studies from germ free-mice have demonstrated that the development of immunity, including mucosal immunity, is dependent on the presence of the resident microbial flora because germ-free mice are missing lymphoid tissues, such as Peyer patches and mesenteric lymph nodes. In contrast, specific pathogen-free mice are depleted of only certain bacterial species.

IL-3: IL-3 is produced by T cells, as well as keratinocytes and mast cells. IL-3 promotes histamine release and causes the differentiation and proliferation of mast cells and basophils.

IL-18: IL-18 is an IL-1 family member that has been shown to have effects similar to IL-12 and induces IFN-γ production. IL-18 has been shown to activate murine basophil populations, and human basophils express the IL-18 receptor.

IL-33: IL-33 is an IL-1 family member that is produced by epithelial cells, smooth muscle cells, and fibroblasts and has been shown to activate human and murine basophil populations.

MAST CELL PROTEASE 8 (Mcpt8): Mcpt8 is exclusively expressed by murine basophils. Transgenic mice expressing yellow fluorescent protein under the control of the Mcpt8 promoter allow isolation of basophils by using cell sorting for yellow fluorescent cells. Furthermore, Mcpt8 has been used to selectively express the diphtheria toxin receptor in murine basophil populations.

PLATELET-ACTIVATING FACTOR: Platelet-activating factor is a mediator of anaphylaxis. The blood levels of platelet-activating factor are increased after anaphylaxis, and the levels correlate with the severity of the anaphylactic reaction.

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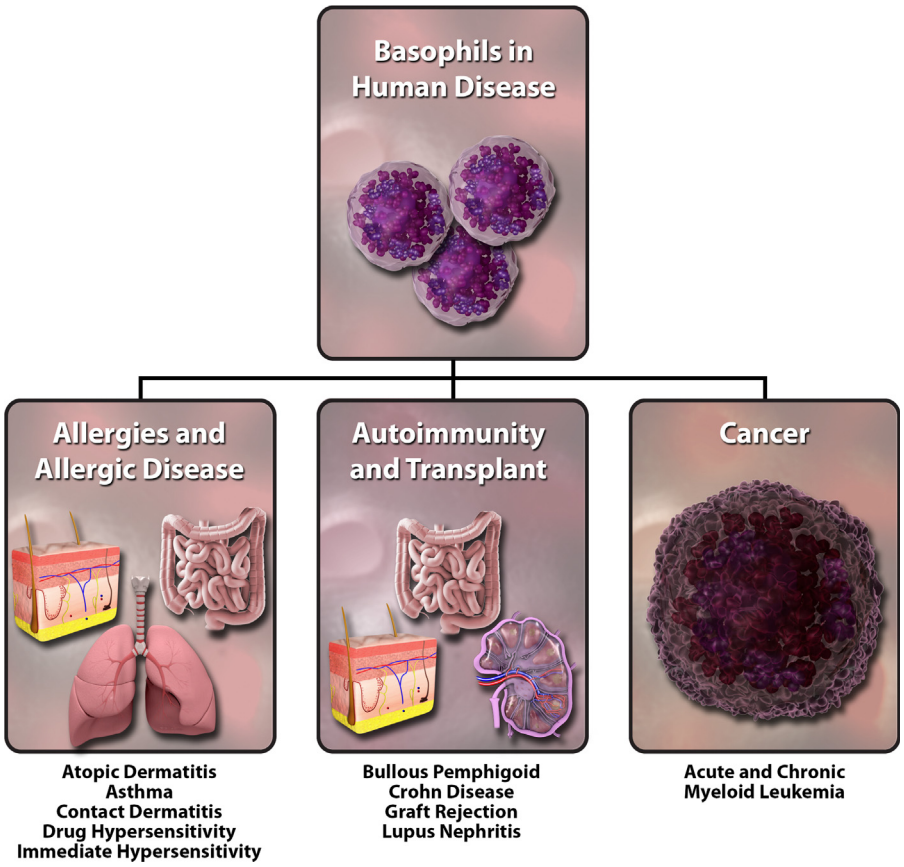

FIG 1. The clinical relevance of basophils. Basophils have been shown to contribute to many human disease states, including allergic diseases (contact dermatitis, AD, hypersensitivity responses, and asthma), autoimmunity (bullous pemphigoid and lupus nephritis), inflammatory disorders (Crohn disease), and cancer (acute and chronic myelogenous leukemia). This article will focus on the contribution of basophils to allergic disease.

and allowed basophils to be identified *in vivo*, systematically phenotyped, and easily tracked using flow cytometric techniques.[31-33] Research from the laboratories of Karasuyama and colleagues and Kubo and colleagues extended these studies to develop novel techniques to deplete murine basophils, thereby allowing the *in vivo* functions of basophils to be tested by targeting the membrane glycoprotein ***CD200R*** or by engineering basophils to express the ***diphtheria toxin receptor*** under the control of basophil-specific IL-4 enhancer elements or proteases.[34-36] In addition, Ohnmacht et al[37] developed a mouse that expressed toxic levels of Cre recombinase under the basophil-specific protease ***mast cell protease 8 (Mcpt8)***, resulting in a loss of greater than 90% of mature basophil populations. Furthermore, Sullivan et al[38] developed Basoph8 mice, which have the Mcpt8 gene replaced with yellow fluorescent protein, allowing for 2-photon imaging of basophil responses *in vivo*. Using these mice, research from Sullivan et al[38] allowed tracking of basophil populations *in vivo* and identified them in both secondary lymphoid tissues and inflamed tissues. Collectively, the ability to ablate, temporally deplete, and track basophil populations *in vivo* has facilitated a series of studies that have directly interrogated the ability of basophils to contribute to the development of T_H2 cytokine–mediated inflammation in murine model systems.[35,37-45] As discussed below, studies using these new mouse models have revealed that basophils function as important contributors to the development of protective immunity to *Trichinella spiralis*,[43] secondary *Nippostrongylus brasiliensis* infection,[46] and immunity to ectoparasites.[34] Furthermore, similar studies demonstrated an important role for basophils in the induction of optimal T_H2 cytokine–mediated inflammation in the context of acute AD, chronic IgE-mediated dermatitis, airway inflammation, and eosinophilic esophagitis (EoE)–like disease.[35,37,42,45,47] Collectively, these new tools and approaches are revealing previously unappreciated roles of basophils in regulating immunity and inflammation.

BASOPHIL DEVELOPMENT

Murine basophils are myeloid in origin and are thought to develop primarily from hematopoietic stem cell (HSC) populations that reside in the bone marrow. However, many of the cellular and molecular events that promote basophil commitment from HSCs remain unknown. This section will describe the known mechanisms that regulate the basophil cell lineage commitment and will discuss how these pathways result in phenotypically and functionally distinct basophil populations that might contribute to allergic disease.

Stem cell populations

Basophils are reported to develop from common HSC-derived granulocyte-monocyte progenitor (GMP) cells that reside in the bone marrow (Fig 2).[48] GMP cells maintain the capacity to

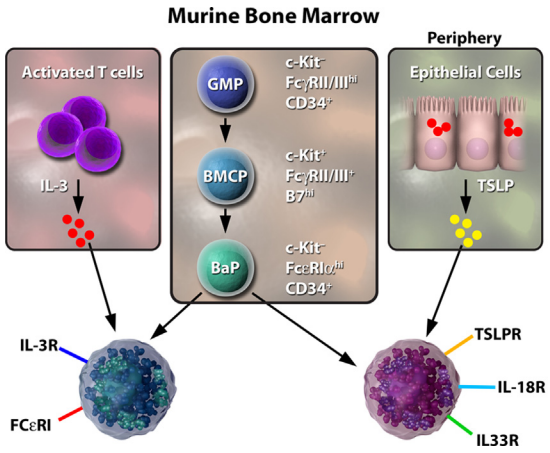

FIG 2. Basophil development. Murine basophils develop from HSC populations that reside in the bone marrow. As HSCs mature, they become GMPs, basophil mast cell precursors *(BMCPs)*, and BaPs. To date, 2 cytokines, T cell–derived IL-3 and epithelial cell–derived TSLP, have been shown to promote distinct phases of basophil development. After fully maturing in the bone marrow, basophils can enter the periphery and contribute to inflammation.

develop into multiple cell lineages, including macrophages, eosinophils, mast cells, and basophils.[48] Critically, as GMP cells mature, they are known to enter intermediate commitment steps and can become mast cell precursors (MCPs), basophil MCPs, or basophil precursors (BaPs; Fig 2).[20,48] Both basophil MCPs and BaPs possess the capacity to develop into mature basophil populations.[48,49] Unlike mast cells, which are known to exit the bone marrow with an immature phenotype and complete their maturation in peripheral tissues, basophils are reported to exit the bone marrow once they have fully matured (Fig 2).[48,49] However, recent studies have identified that multiple bone marrow–resident progenitor cell populations, including GMP cells, exit the bone marrow in the context of inflammation and undergo extramedullary hematopoiesis (EMH) in the periphery.[50,51] Although this process remains to be fully defined, it is likely that the peripheral basophilia observed in the context of T_H2 cytokine–mediated inflammation is supported by the development of basophils from bone marrow–resident cells and through EMH. However, additional studies are needed to further determine the contributions of EMH and basophil development in promoting inflammation.

Transcription factors

Although the commitment of progenitor cells to the basophil cell lineage remains to be fully defined, several binding proteins and transcription factors are known to play critical roles in the process. For example, mature basophil development is reported to be dependent on the expression of ***C/EBPα*** and ***GATA-2***.[49] In addition, recent studies by Mukai et al[52] demonstrated that mice deficient in the transcription factor distal promoter Runt-related transcription factor 1 (P1-Runx1) have a 90% reduction in mature basophil populations in the periphery but exhibit normal numbers of neutrophils, eosinophils, and mast cells. Collectively, these studies identify P1-Runx1 as a selective regulator of basophil development in mice.

Environmental factors

Recent studies have also identified that beneficial microbial communities, including commensal bacteria, can have significant effects on basophil development and activation.[53,54] For example, eliminating or experimentally altering commensal bacteria–derived signals resulted in increased serum IgE levels in ***germ-free mice*** or antibiotic-treated mice compared with those seen in conventionally housed mice.[53,54] Increases in IgE levels promoted the development of mature basophil populations by enhancing the responsiveness of progenitor cell populations to growth factors.[53] Consistent with murine studies, it was also shown that increased IgE levels in immunodeficient patients with atopic disorders were associated with increased frequencies of circulating basophils.[53] Collectively, these data indicate that commensal microbial-derived signals and IgE regulate basophil development. Given the established association between repeated exposure to antibiotics during childhood and the development of allergic inflammation,[55,56] it is tempting to speculate that dysregulated basophil responses might contribute to these processes.

Cytokines

Unlike the lifespan of other granulocyte populations, the lifespan of mature basophils is relatively short and estimated to be between 1 and 2 days.[49,57,58] Therefore the constant presence of basophils in the periphery is thought to be a result of continuing development and replenishment of cells from bone marrow–resident progenitors.[57] In the context of T_H2 cytokine–mediated inflammatory responses, increased basophil development and peripheral basophilia are often observed,[35,42,43,59-61] suggesting that basophil development can be positively regulated by proinflammatory factors. Seminal studies by Lantz et al,[59,60] Shen et al,[61] and Ohmori et al[62] demonstrated that peripheral basophilia after *N brasiliensis* or *Strongyloides venezuelensis* infection is critically dependent on ***IL-3***–IL-3 receptor signaling. These studies provoked the hypothesis that peripheral basophilia was predominately regulated by IL-3 signaling (Fig 2). This hypothesis was further supported by subsequent reports demonstrating that basophil recruitment to the draining lymph nodes after *N brasiliensis* infection was IL-3 dependent.[63]

Although it is clear that IL-3 is a key regulator of basophil development in the context of some stimuli, recent studies have identified that the predominantly epithelial cell–derived cytokine thymic stromal lymphopoietin (TSLP) also regulates basophil development and peripheral basophilia (Fig 2).[42,43] For example, it was demonstrated that peripheral basophilia after *Trichuris muris* infection, *Trichinella spiralis* infection, or induction of AD-like inflammation is critically dependent on TSLP–TSLP receptor signaling.[42,43] In addition, it was determined that TSLP was capable of cooperating with IL-3 to promote optimal basophil responses but also maintained the capacity to promote basophil development and peripheral basophilia in the absence of IL-3–IL-3 receptor signaling.[42] Critically, TSLP-elicited basophils exhibited distinct phenotypic and functional characteristics from classical IL-3–elicited basophils. Most notably, they lacked the ability to degranulate in response to IgE-mediated FcεRI signaling but were potent producers of IL-4 in response to IL-3, ***IL-18***, or ***IL-33*** stimulation.[42] Collectively, these studies demonstrate that basophil responses can be regulated by IL-3/IgE–dependent or TSLP-dependent mechanisms (Fig 2, see below).

As described above, recent data suggest that there is an IL-3–elicited basophil population that is activated by IgE and a

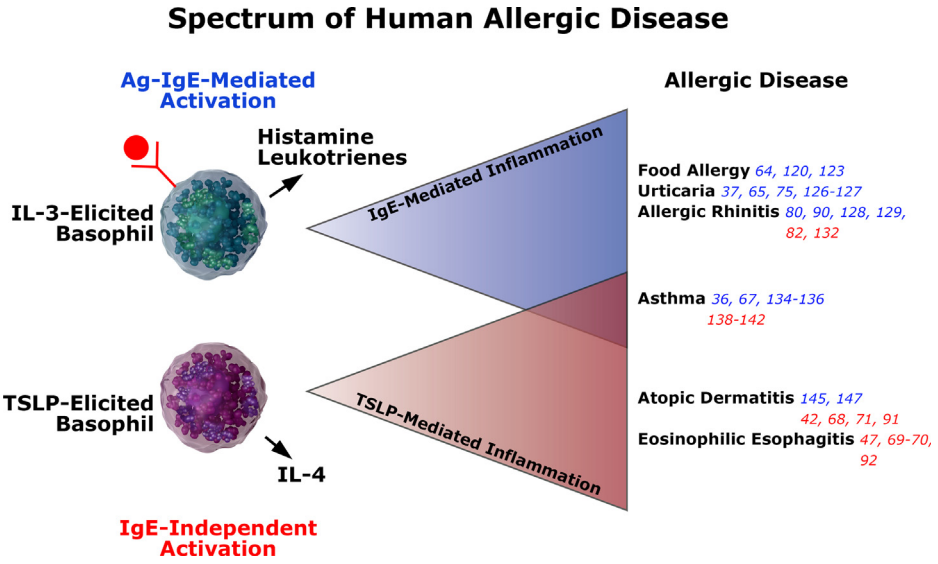

FIG 3. Heterogeneous effector functions of basophil populations. Recent studies have demonstrated that IL-3–elicited basophils are highly responsive to FcεRI cross-linking through IgE-antigen complexes. After encountering antigen, IL-3–elicited basophils release multiple effector molecules that can contribute to allergic inflammation. TSLP-elicited basophils are nonresponsive to IgE-antigen complexes but produce multiple effector molecules after stimulation with cytokines, such as IL-33 and IL-18. The observed functional heterogeneity between IL-3–elicited and TSLP-elicited basophils might allow IL-3–elicited basophils and TSLP-elicited basophils to contribute to various allergic disorders that are associated with IgE responses, TSLP production, or both.

distinct TSLP-elicited basophil population that appears to function independently of IgE (Fig 2).[42] In this review we will focus on how these distinct pathways and cell types might contribute to various allergic disease states. The recent identification of functionally distinct basophil populations is of particular interest to our understanding of human allergic disease states, such as food allergy and EoE, asthma, urticaria, allergic rhinitis (AR), and AD. Some conditions, such as food allergy,[64] urticaria,[65,66] and asthma,[67] can be predominately IgE-mediated and responsive to anti-IgE therapy, whereas others, such as AD[68] and EoE,[69] have shown mixed results in response to anti-IgE therapy, indicating that IgE-independent processes might be critical for the pathogenesis of these diseases (Fig 3).[35,37,47,64,66,67,70-90] Furthermore, AD and EoE have been directly associated with polymorphisms in *TSLP* and increased TSLP protein production at the site of inflammation.[70,71,91,92] These observations provoke the hypothesis that there might be IgE-dependent basophil responses that contribute to inflammation in some diseases states (eg, food allergy, urticaria, and asthma) and TSLP-elicited, IgE-independent basophil responses that contribute to inflammation in other disease states (eg, AD and EoE; Fig 3). Whether these pathways represent heterogeneous mechanisms across allergic disease states or within one disease remains an active area of investigation. In the sections below, we will discuss in more detail the potential interplay between IgE, TSLP, and basophil function in human disease.

In summary, there are a variety of developmental, transcriptional, and cytokine-mediated pathways that can influence the function of basophils. The following sections will put these different aspects into the context of potential basophil heterogeneity as it pertains to human allergic disease.

BASOPHIL ACTIVATION AND EFFECTOR FUNCTIONS

Basophils can be activated by an array of signals, including those mediated by cytokines, antibodies, proteases, and directly by antigens themselves. The following section will highlight the known mediators of basophil activation and describe the effector processes they initiate. We will then describe how these distinct methods of activation and effector mechanisms are associated with human allergic disease states and might promote the development of allergic inflammation.

Antibody-mediated activation

Perhaps the best studied and most well-recognized mode of basophil activation is initiated through FcεRI and IgE-mediated cross-linking (Table I).[31,40,42,60,85,94-110] Basophils have been shown to produce effector molecules, such as histamines and leukotrienes, in response to IgE-mediated activation.[16] The ability of basophils to produce histamines and leukotrienes in response to IgE-antigen complexes has implicated these cells in the induction of smooth muscle contraction and as contributors to systemic anaphylaxis (Table I).[111] Although basophils have never been directly shown to contribute to IgE-mediated anaphylaxis in experimental systems, it has been reported that basophils promote an alternative pathway of anaphylaxis in response to IgG-antigen complexes in C57BL/6 mice.[93] Specifically, basophils have been shown to produce ***platelet-activating factor*** and significantly contribute to anaphylaxis in response to penicillin-IgG antibody complexes.[93] In addition to IgE- and IgG-mediated activation, basophils have also been shown to be activated in an IgD-dependent manner. For example, IgD-antigen complexes can induce the production of antimicrobial peptides from basophils, and supernatants from IgD-activated basophils were capable of inhibiting the

TABLE I. Summary of the activating factors and effector molecules of murine and human basophils

	Mouse			Human		
Stimulus	**Response**	**Effector molecules**	**Reference**	**Response**	**Effector molecules**	**Reference**
Immunoglobulin						
IgD	?			Yes	IL-1, IL-4, antimicrobial peptides, B-cell activating factor	94
IgE	Yes	IL-4, histamine	102,103	Yes	IL-4, IL-13, histamine, leukotriene, platelet-activating factor	95,96,104
IgG	Yes	Platelet-activating factor	93	No		
Cytokine						
IL-3	Yes	IL-4, IL-13	105	Yes	IL-13, histamine, leukotriene, amphiregulin	85,106,107
IL-18	Yes	IL-4, IL-13, histamine	98	?		
IL-33	Yes	IL4, IL-13	98,105	Yes	IL4, IL-5, IL-6, IL-13	97
TSLP	Yes	IL-4, IL-6, CCL3, CCL4, CCL12 Cxcl12	42	?		
Complement						
C5a	?			Yes	Histamine	108,109
Allergen						
Der p 1	?			Yes	IL-4, IL-5, IL-13	99
Papain	Yes	IL-4	40	?		
Microbial						
Necator americanus	?			Yes	IL-4, IL-5, IL-13	99
Nippostrongylus brasiliensis	Yes	IL-4	31	?		
Strongyloides venezuelensis	Yes	IL-4	60	?		
Schistotome-derived IPSE/α-1	Yes	IL-4	101	?		
HIV-derived GP120	?			Yes	IL-4, IL-13	100
TLR ligands	?			Yes	IL-4, IL-13	111

?, Unknown or untested areas of research.
TLR, Toll-like receptor.

growth of certain bacteria.[94] Collectively these findings show that the humoral immune system influences basophil function via distinct pathways. However, further studies are needed to better understand the kinetics of IgG-, IgD-, and IgE-mediated basophil activation and the specific mediators that are released by both IL-3– and TSLP-elicited basophils in response to these distinct stimuli.

Cytokines

As discussed above, IL-3 is capable of promoting basophil development both *in vitro* and *in vivo*; however, in addition to its effects on basophil development, IL-3 is capable of promoting basophil activation. For example, IL-3 can directly promote the release of cytokines (IL-4 and IL-6) and chemokines (CCL3, CCL4, CCL12, and Cxcl2) from TSLP-elicited basophil populations in an IgE-independent manner in mice (Table I)[16,42] and can also enhance IL-4 and IL-13 production from human basophils after IgE-mediated activation.[95,96] In addition to IL-3, the IL-1 cytokine family members IL-18 and IL-33 can also directly activate basophils and enhance their effector functions.[42,97,98,112-114] In both mice and humans IL-33 has been shown to activate basophils, but IL-18 has only been demonstrated to activate murine basophils (Table I). For example, IL-18 treatment promotes the production of IL-4 from basophil populations in a myeloid differentiation primary response gene (88; MyD88)–dependent manner.[98] Similar to IL-18, IL-33 also promotes IL-4 and IL-13 production from basophil populations in a MyD88-dependent manner.[98] Critically, although IL-3–elicited basophils degranulate in response to IgE-mediated cross-linking,[42] they did not respond robustly to IL-3, IL-18, or IL-33 stimulation.[42] In contrast, although TSLP-elicited basophils exhibit a limited ability to degranulate in response to IgE-mediated cross-linking, they respond robustly to stimulation with IL-3, IL-18, or IL-33.[42] Collectively, these studies highlight that IL-3–elicited and TSLP-elicited basophils differentially respond to IgE-mediated activation and are further distinguished by their response to a variety of cytokines.

Direct activation by protease allergens

In addition to antibody- and cytokine-mediated activation, basophils respond directly to protease activity. For example, the house dust mite (HDM) protease Der p 1 can promote the production of IL-4, IL-5, and IL-13 from human basophil cell lines (Table I).[99] Furthermore, parasite-derived proteases and the cysteine protease papain have been shown to promote the production of type 2 cytokines from murine basophils.[40,99] These studies also demonstrated that rendering the proteases inactive through exposure to heat inactivation or treatment with protease inhibitors eliminated their ability to promote basophil activation, suggesting that basophils are sensing the protease activity directly. Collectively, these data suggest that basophils are capable of sensing and responding to active proteases, but the mechanisms through which this occurs remain unknown. Moreover, the ability of basophils to detect proteases might allow them to respond robustly to common allergens, many of which possess protease activity.

Additional mediators of activation

Basophils are known to respond to a variety of environmental stimuli, such as drugs, venoms, and pollens, and their reactivity can be assessed by using the BAT.[12] Additionally, human

peripheral blood basophils have been shown to spontaneously release histamine in response to a histamine-releasing factor from other mononuclear cell populations in patients with food allergy and AD.[115,116] A less well-understood method of basophil activation is initiated by a series of "superantigens" that are capable of promoting basophil activation independently of antigen-specific antibodies or protease activity (Table I). For example, the gp120 glycoprotein of HIV is capable of nonspecifically interacting with surface-bound IgE on human basophils and promoting IL-4 and IL-13 production.[100,117] Similarly, the schistosome-derived glycoprotein IPSE/α-1 also promotes IL-4 production by basophils in the absence of antigen-specific IgE.[101] However, whether common allergens can also act as superantigens and promote type 2 cytokine production by basophils remains unknown. Collectively, these findings demonstrate that a variety of naturally occurring and pathogen-associated stimuli can directly influence basophil function.

Additional effector mechanisms

Additional studies have also shown that MHC class II^+ basophils can promote T_H2 cytokine–associated inflammation through antigen presentation to T cells.[39,41,44] Although murine basophils have been reported to function as antigen-presenting cells (APCs), subsequent studies investigating the role of basophils as APCs in human subjects have been less clear.[72,73,118,119] Therefore future studies are required to determine the clinical significance of basophils functioning as APCs, as the discovery of therapies targeting dendritic cells is an active area of investigation in patients across multiple diseases.

BASOPHILS AND ALLERGIC INFLAMMATION: PATHOGENESIS AND IMPLICATIONS FOR CLINICAL MANAGEMENT

Classically, allergic or atopic diseases are driven by T_H2 cytokine responses and therefore are associated with the production of IL-4, IL-5, IL-9, and IL-13. Furthermore, the inflammatory responses underlying these conditions are associated with peripheral eosinophilia, IgE production, and tissue-resident mast cell responses.[74] In recent years, basophils have emerged as contributors to the pathogenesis of multiple models of allergic disease.[35,37,42,75] In the following sections we will discuss experimental evidence obtained by using murine model systems of human allergic diseases to illustrate the potential of targeting basophil populations as a therapeutic strategy. In addition, we will highlight the potential role of basophils in the development and progression of human allergic diseases and discuss how current treatment strategies might unintentionally target basophil-specific pathways. Finally, we will illustrate how using more specific methods to target functionally distinct basophils in the context of allergic inflammation might result in increased therapeutic potency.

Food allergy

Allergic reactions to food remain the leading cause of anaphylaxis that results in emergency department visits.[120] Although anaphylaxis has a much lower incidence (0.03% to 2%) than other allergic conditions, such as AD, it is a life-threatening condition.[121] Anaphylaxis in the context of food allergy is mediated by antigen-specific IgE responses to ingested food allergens.[121] Although food allergy can be prevented by avoiding known food allergens, immunomodulatory therapeutics to prevent the onset of symptoms are limited. Recent clinical studies indicate that anti-IgE therapy with omalizumab might be a useful therapeutic approach in the treatment of food allergy and anaphylaxis by targeting IgE-mediated release of various proinflammatory factors.[64,122] Although the precise contribution of the IgE-basophil axis in anaphylaxis and food allergy remains poorly defined, a recent study using omalizumab in patients with peanut allergy identified that early clinical responses to therapy correlated with basophil suppression rather than mast cell suppression.[122] Furthermore, the BAT is a clinical tool used to test the IgE-mediated reactivity of basophils to food allergens.[123] These studies indicate that targeting IgE-FcεRI interactions on basophils might represent a promising new method to treat food allergy and anaphylaxis. Clinical trials are currently underway to determine whether anti-IgE therapy before desensitization results in faster or safer reductions in allergic reactions.

Although the role of TSLP in classical IgE-mediated food allergy remains unclear, its potential role in the food allergy–associated disease EoE has emerged as an active area of investigation. EoE is characterized by chronic inflammation of the esophagus associated with ingested or inhaled allergens, and in contrast to classical food allergy associated with anaphylaxis, anti-IgE therapy has demonstrated poor efficacy in ameliorating EoE symptoms.[64,69] Although swallowed topical steroid therapy is effective in treating EoE, side effects with regard to long-term steroid use in children are a significant concern, and additional therapeutic approaches would greatly aid in the treatment of EoE.[124] Strikingly, gain-of-function polymorphisms in *TSLP* have been strongly associated with the development of EoE in patients.[70,92] Although the role of basophils remains poorly defined in patients with EoE, recent findings have shown that patients with EoE and a gain-of-function polymorphism in *TSLP* present with basophil populations that exhibit the phenotype of TSLP-elicited basophils in mice.[42] Furthermore, patients with a gain-of-function polymorphism in *TSLP* present with increased peripheral basophilia.[47] These data, along with the ability of TSLP-elicited basophils to promote IgE-independent inflammation, provoke the hypothesis that TSLP-elicited basophils can contribute to the pathogenesis of EoE through a distinct mechanism from the IgE-dependent pathways that contribute to classical food allergy. Further differentiating the role of IgE-activated versus TSLP-elicited basophils in patients with classical food allergy and EoE might provide significant insight into the pathogenesis of these conditions.

These concepts are supported by recent studies using a new murine model of EoE-like disease. Specifically, studies in our laboratory identified that TSLP promotes IgE-independent murine EoE-like disease characterized by eosinophilic inflammation and food impaction after repeated challenges with food antigens.[47] Critically, EoE-like disease was associated with a significant population expansion of TSLP-elicited basophils and T_H2 cytokine responses. Furthermore, depletion of TSLP-elicited basophils before the initiation or after the onset of inflammation in the esophagus was established resulted in a loss of EoE-like disease.[47] Translational studies revealed that patients with EoE had increased expression levels of *TSLP* and

significantly increased basophil populations in esophageal biopsy specimens.[47] Collectively, these findings provoke the hypothesis that TSLP elicits a functionally distinct population of IgE-independent basophils in the context of EoE. Understanding the mechanisms by which TSLP-elicited basophils contribute to the pathogenesis of EoE and how these mechanisms differ from the IgE-dependent mechanisms that promote classical food allergy might provide significant insight toward new therapeutic strategies for these conditions.

Urticaria

Urticaria is a very common skin condition that results in the development of itchy wheals or hives. When this condition lasts longer than 6 weeks, it is referred to as chronic urticaria. Many cases of chronic urticaria lack an identifiable cause and are referred to as chronic idiopathic urticaria (CIU). A significant portion of patients with CIU have recently been shown to have urticaria in response to anti-IgE–FcεRIα antibodies that might activate mast cells or basophils.[125] Furthermore, basophil activation has been associated with urticaria in patients in response to IL-3, as demonstrated by upregulation of CD203.[126,127] Consistent with previous findings that IL-3–elicited basophils are responsive to IgE-mediated activation, murine studies have shown that basophils critically orchestrate IgE-mediated chronic allergic inflammation in the skin.[75] Although CIU is thought to be mediated by both IgE-dependent and IgE-independent mechanisms, a recent study revealed that omalizumab is effective in treating the symptoms of CIU.[66] It is widely appreciated that many patients with CIU do not respond to aggressive first-line therapies with antihistamines. However, Maurer et al[66] demonstrated that patients who were unresponsive to treatment with H_1-antihistamine therapy responded to omalizumab, which is now currently awaiting US Food and Drug Administration (FDA) approval for CIU as a new indication. Furthermore, recent studies indicate that omalizumab might be influencing basophil function,[76,128] but future studies will be required to determine the precise contribution of basophils to urticaria. Although the role of IgE in promoting urticaria is widely appreciated, the role of TSLP and TSLP-elicited basophils in the development of urticaria remains to be determined.

AR

AR affects 40 million persons in the United States across all ethnic, socioeconomic, and age groups.[77] Classically, AR is thought to be mediated by IgE responses to allergenic proteins in the environment and subsequent cross-linking of mast cells. Activation of mast cells results in the release of a variety of inflammatory mediators, such as histamines, leukotrienes (eg, leukotriene C_4 [LTC_4]), and prostaglandin D_2, to promote clinical rhinorrhea.[78] On the basis of these pathophysiologic features, therapies to treat AR include antihistamines, inhaled corticosteroids, lipoxygenase inhibitors (zileuton), leukotriene antagonists (zafirlukast and montelukast), and mast cell stabilizers (cromolyn sodium).[79] Furthermore, given that this condition is thought to be mediated by antigen-specific IgE, omalizumab has been proposed as a potential therapeutic agent.[80] Basophils have been identified in the nasal washes of patients with AR and are thought to be the dominant source of histamine in late-phase responses to allergen challenge in patients.[81,129] Furthermore, as noted above, basophils are a significant source of LTC_4. Thus therapeutics that are thought to target mast cell activation in the context of AR might partially be deriving their efficacy based on their effect on basophil-derived histamine and leukotrienes.

Despite these advances, the role of IgE-activated basophils in patients with AR remains poorly understood and an area of active investigation. For example, recent studies indicate that patients with AR can demonstrate local IgE responsiveness to allergens in the absence of systemic IgE-based reactions.[130] Therefore whether basophils act locally or systemically in patients with AR remains an area that can yield significant insight. In addition to IgE-based studies, a recent genome-wide association meta-analysis of patients with AR demonstrated an association with *TSLP* variants,[131] and subsequent studies identified increased expression of *TSLP* in nasal polyps, which are strongly associated with AR.[82,132] Although these findings are supportive of a causative role for TSLP in the sequelae of AR, future studies are required to determine whether IgE-activated basophils or TSLP-elicited basophils contribute to the pathogenesis of AR.

Asthma

Asthma affects 300 million people worldwide and is the most common chronic disease of childhood.[133] Currently, therapeutics in asthma include β-agonists, oral and inhaled corticosteroids, anticholinergics, phosphodiesterase inhibitors, molecules that inhibit leukotriene production (zileuton, zafirlukast, and montelukast), and anti-IgE mAb (omalizumab).[83,84] Omalizumab is the first anti-IgE therapy to demonstrate efficacy and be approved by the FDA for asthma.[67] Basophils activated by IgE are known to release histamine and LTC_4 to promote inflammation.[18] Despite the fact that basophil-associated pathways are targeted by some of these therapeutics, the precise role of basophils in the pathogenesis of asthma remains poorly understood.

Animal model systems have provided some insights into potential roles for basophils in contributing to the development or propagation of allergic airway inflammation. For example, in a recent study using a murine model of HDM-induced airway inflammation, basophils were found to play a direct role in promoting optimal T_H2 cytokine responses.[53] Although it was demonstrated that a rare population of FcεRI-expressing inflammatory dendritic cells was found to be both necessary and sufficient for the development of airway inflammation, specific depletion of basophils after the induction of airway inflammation resulted in significantly reduced T_H2 cytokine–associated inflammation.[45] Collectively, these studies suggest that basophils might cooperate with dendritic cell populations to contribute to pathologic airway inflammation.[45] Although the contribution of basophils to the pathogenesis of asthma in human subjects remains poorly understood, studies have identified that basophils are highly enriched in postmortem lung tissue of patients who have died from asthma, as well as in bronchial biopsy specimens of patients with asthma.[5,6] Furthermore, a recent study identified that T cell–derived IL-3 induces the expression of amphiregulin from human basophils.[85] Although amphiregulin has recently been shown to be a critical growth factor for the orchestration of epithelial repair and remodeling in the airway, the role of basophil-derived amphiregulin in asthmatic patients remains poorly defined.[86] These

findings suggest that basophils might contribute to the pathogenesis of asthma in human subjects, but future studies will be required to directly address this hypothesis.

Classically, histamine and other inflammatory factors derived from IgE-activated mast cells are thought to be the primary mediators of asthma-associated inflammation.[87] Furthermore, increased FcεRI expression has been shown to reduce innate immunity to rhinovirus, the most common trigger of asthma flares.[88] The role of IgE in the pathogenesis of asthma was further reinforced by the finding that anti-IgE therapy has demonstrated efficacy in patients with high serum IgE levels.[67] Although we are only starting to understand the cellular and molecular mechanisms by which anti-IgE therapy mediates its beneficial effects, one possibility is that blocking IgE disrupts IgE-mediated activation of basophils and the release of basophil-derived histamine, LTC_4, and other inflammatory mediators. Indeed, omalizumab therapy has been shown to correlate with reduced basophil FcεRI expression[89,90,134,135] and reduced allergen-mediated basophil activation.[128,134-137] Despite these advances, the specific role of the IgE-basophil axis in asthmatic patients remains to be determined.

In addition to IgE-mediated basophil responses, recent murine studies have demonstrated a critical role for TSLP–TSLP receptor interactions in promoting inflammation in different animal models of airway hyperresponsiveness.[138,139] For example, a recent study using a murine model of HDM-induced allergic inflammation in the lung demonstrated that TSLP blockade ameliorates disease.[138] In support of its role in human asthma, gain-of-function polymorphisms in *TSLP* have been associated with asthma and allergic airway disease in patients.[140] Furthermore, TSLP signaling was shown to promote asthmatic airway remodeling pathways in human lung fibroblasts, and its expression was found to be significantly increased in bronchial biopsy specimens from patients with severe asthma.[141,142] Despite these developments, the cellular mechanisms by which TSLP promotes allergic inflammation in the lung and whether TSLP-elicited basophils play a role in asthma pathogenesis remain to be determined.

Asthma is a disease that is phenotypically and pathophysiologically heterogeneous in its clinical presentation and response to treatments.[143] Prior studies have shown that asthmatic patients exhibit phenotypically distinct basophil populations in the peripheral blood, some of which respond robustly to IgE-mediated activation, whereas others are minimally responsive.[144] As such, uncovering the precise roles of IgE-activated versus TSLP-activated basophils might help to clarify the complex inflammatory mechanisms that underlie asthma.

AD

AD is a chronic relapsing skin disease that is associated with the development of food allergies, asthma, AR, and urticaria. It often begins in the first year of life and affects as many as 20% of children and 2% to 9% of adults in the US.[74] AD has classically been associated with T_H2 cytokine responses and increased serum IgE levels in patients. However, the precise role of these pathways in AD remains poorly defined. It has been observed that, early in infancy, IgE responses are not present because of an immature adaptive immune system, but subsequent sensitization to food and environmental allergens results in the development of allergen-specific IgE.[74] Furthermore, a recent study showed that antigen-specific IgE-mediated activation of basophils occurs in the peripheral blood of patients with AD.[145] In a similar context, IgE-dependent basophils were also found to be critical for the pathogenesis of chronic AD in mice in an IgE-dependent manner.[37] Although basophils only accounted for a small proportion of the cellular infiltrate found in the lesional skin, depletion of basophils resulted in a significant reduction in numbers of infiltrating eosinophils and neutrophils and also resulted in a dramatic loss in skin thickness.[37] Whether these mouse models are more representative of AD or urticaria remains to be determined. Collectively, these studies provoke the hypothesis that IgE-activated basophils might play a role in AD.

Although basophils have recently been implicated in the pathogenesis of murine AD-like disease, their precise role in human disease remains to be defined. The development of novel mAbs (J175-7D4, BB1, and 2D7) that specifically stain human basophils has allowed for the identification of basophils in the lesional skin of patients with AD by using immunohistochemical techniques (Kim et al, unpublished data).[2,146] Using anti-human basophil-specific mAb (2D7) and flow cytometric techniques, we have also identified enrichment of human basophils in the lesional skin of patients with AD but not in healthy control subjects or control psoriatic skin (Kim et al, unpublished data). Although these studies provoke the hypothesis that basophils can contribute to human AD, whether IgE-activated or TSLP-elicited basophils predominate in human AD remains unclear.

In recent years, TSLP has been associated with human AD and identified as an early promoter of T_H2 cell–associated cytokine responses.[71] Furthermore, TSLP has been found to be a key hematopoietic cytokine in the elicitation of functionally distinct basophil populations that are potent producers of IL-4 and are activated independently of IgE.[42] In a murine model of AD associated with increased TSLP production, significant TSLP-dependent basophilia was observed in lesional AD-like skin.[42] Critically, specifically targeting and eliminating TSLP-elicited basophil populations reduced T_H2 cytokine responses in the skin-draining lymph nodes, suggesting that TSLP-elicited basophils contribute to the pathogenesis of AD-like inflammation. These findings are consistent with the finding that many patients with AD, particularly infants, can have disease that is promoted primarily by TSLP and its direct effects on the innate immune response as opposed to antigen-specific IgE and adaptive immune responses. However, the precise role of TSLP-elicited basophils in human AD remains to be determined.

Topical steroids remain the first-line agents in the treatment of AD. However, these treatments are associated with a wide range of both local and systemic side effects of particular concern in children.[147] Although there are no therapies designed to specifically target basophils, there are a number of therapeutics that target pathways common to both basophils and other hematopoietic cells. For example, the only nonsteroidal anti-inflammatory FDA-approved agents in patients with AD are topical calcineurin inhibitors, tacrolimus, and pimecrolimus. Although these agents are thought to act on T cells by inhibiting nuclear factor of activated T cell family–mediated transcriptional activation of IL-2, previous studies have shown that calcineurin inhibitors prevent IgE-mediated activation of IL-4 production through nuclear factor of activated T cell pathways in human basophils.[148,149] These findings suggest that existing therapeutics

in patients with AD might actually modulate basophil-specific pathways.

Although omalizumab blocks IgE-FcεRI interactions on both mast cells and basophils, clinical studies with omalizumab have yielded mixed results in patients with AD.[68] AD is a disease with complex diagnostic criteria in which the role of IgE remains poorly understood. Therefore future studies with well-defined stratification of disease might be required to address the role of IgE and basophils in patients with AD. Additionally, recent studies implicating TSLP as a key mediator of basophil hematopoiesis offer a new avenue of investigation.[42] Future studies specifically targeting TSLP, basophils, or both might provide new therapeutic targets for novel biologic agents to treat AD. Elucidating whether IgE-activated basophils, TSLP-elicited basophils, or both contribute to the pathogenesis of AD will help to direct the development of future therapeutics.

INFLUENCE OF ANTI-IGE THERAPY ON BASOPHILS

The growing number of clinical trials using anti-IgE therapy has resulted in the emergence of a better understanding of the role of IgE in regulating basophil responses. For example, a number of studies have shown that anti-IgE therapy results in the downregulation of FcεRIα expression on basophils,[89,134,150] as well as a reduction in basophil effector responses during anti-IgE therapy.[136,137] However, the precise mechanisms by which anti-IgE therapy results in reduced basophil responses and whether these effects contribute to the clinical improvement observed with anti-IgE therapy remain to be determined. Nonetheless, a better understanding of these pathways might identify biomarkers of disease severity or allow for the development of new targeted approaches for the treatment of allergic disease.

CONCLUDING REMARKS

Basophils are implicated in multiple human diseases, including autoimmune disorders, inflammatory disorders, cancer, and allergies and asthma. However, the contributions of basophils to the development of human disease states remain poorly defined. Recent murine and human studies suggest that developmental and functional heterogeneity exists within basophil populations and that basophils can be divided into at least 2 categories: IL-3–elicited basophils and TSLP-elicited basophils (Fig 3). Moreover, these studies suggest that IL-3–elicited basophils operate in an IgE-dependent manner, whereas TSLP-elicited basophils operate in an IgE-independent manner. It is also becoming more apparent that allergic conditions can also be stratified into 2 categories: those mediated by IgE and those that appear to be IgE independent. Critically, allergic diseases that are thought to be predominately IgE independent are highly associated with gain-of-function polymorphisms in the gene encoding *TSLP* and increased TSLP expression (Fig 4). Therefore it is likely that the contributions of basophils to human allergic disorders will differ depending on the allergen, the disease state, and whether the disease is IgE dependent or TSLP dependent. These recent studies might also provide insight into the varied efficacy of anti-IgE therapy and other biologic agents across different allergic disease states. Although anti-IgE treatment might be beneficial in preventing the activation of IL-3–elicited basophil populations, this strategy could prove ineffective in targeting TSLP-elicited basophils. Thus it is likely that directly targeting basophil populations or simultaneously targeting both TSLP and IgE might prove beneficial in the treatment of allergic disease states. Currently, clinical trials are underway using anti-TSLP mAbs in patients, and multiple studies are investigating the influence of anti-IgE therapy on basophil populations in patients (see www.clinicaltrials.gov). Future studies of basophil phenotype, activation, and function in patients undergoing anti-TSLP and anti-IgE treatment would yield significant insight into the clinical relevance of basophil heterogeneity in the context of human allergic disease.

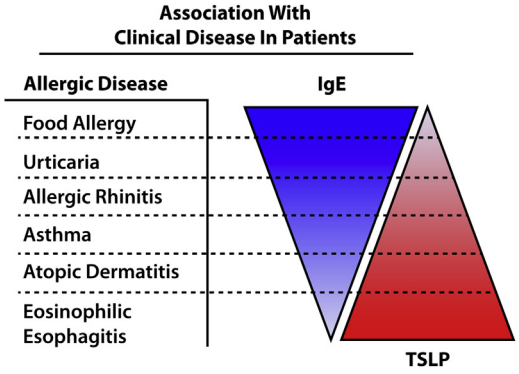

FIG 4. The contributions of IgE and TSLP to allergic disease. Allergic disease states appear to separate into 3 separate categories: those that are highly IgE dependent (urticaria and food allergy), those that are partially IgE dependent (asthma and AD), and those that are IgE independent (eosinophilic esophagitis). Critically, allergic diseases that are only partially dependent on IgE or independent of IgE are highly associated with TSLP production. Therefore it is likely that IL-3–elicited basophils that are highly responsive to IgE-antigen complexes contribute to IgE-mediated allergic disorders, whereas TSLP-elicited basophils contribute to IgE-independent disorders.

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Relating microarray component testing and reported food allergy and food-triggered atopic dermatitis: a real-world analysis

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ABSTRACT

Background: High epitope diversity has been associated with increased IgE-mediated food allergy severity.
Objective: To characterize associations between results from an automated microarray system and self-reported food allergy and food-triggered atopic dermatitis (AD).
Methods: Families with food allergic children were identified from a Jewish community in Lakewood, New Jersey, with immediate family members without food allergy or food-triggered AD serving as controls for the identified children. Sets of microarray components analyzed were to milk (Bos d 4, Bos d 5, Bos d 8, Bos d lactoferrin), egg (Gal d 1, Gal d 2, Gal d 3, Gal d 5), and peanut (Ara h 1, Ara h 2, Ara h 3, Ara h 6).
Results: Seventy-three patients from 23 families were recruited. Culprit foods included milk (n = 20), egg (n = 10), and peanut (n = 6) for food allergy and milk (n = 10) and egg (n = 7) for food-triggered AD. Odds of having had a self-reported related food allergy or food-triggered AD reaction significantly increased with a higher number of detectable microarray components to that food. Ara h 1, Ara h 2, and Ara h 6 were individually associated with reported peanut allergy, and Bos d 4 was individually associated with reported milk allergy. The number of egg components significantly increased the odds of having related food-triggered AD.
Conclusion: High diversity of food allergen components relates well to self-reported history of food allergy and food-associated AD.

Introduction

Food allergy is a common disease that affects 2% to 10% of the population.[1–4] The criterion standard for diagnosis is a double-blind, placebo-controlled food challenge. However, this challenge is not always performed because of resource unavailability and potential reaction risk. More commonly, a food allergy diagnosis is made based on clinical history combined with skin prick test (SPT) and/or fluorescence enzyme immunoassay (FEIA). These investigations have their limitations. Although both SPT and FEIA have a high sensitivity of greater than 85%, specificity is lower at 40% to 80%.[5] FEIA can yield a 95% positive predictive value in some foods[6]; however, because whole food extracts are used in FEIA, results may be falsely positive if the patient generates IgE antibodies against cross-reactive allergens or epitopes within the food that are not clinically relevant to inducing an allergic reaction. In patients with atopic dermatitis (AD), test result interpretation can be particularly difficult. No clinical decision points have been identified for eczematous reactions.[7] Although eczematous reactions can be seen on food challenge in children with AD, in one study the FEIA sensitivity and specificity were low at 68% and 50%, respectively.[8]

Component-resolved diagnosis by microarray is a novel method of analyzing food allergy. A specific panel of purified or recombinant protein components for each food extract is provided within a microarray, and detection for host-specific IgE antibody to these proteins is identified. Microarray assessment permits quantitative data to be collected in a robust fashion, which may help better characterize food allergy. In addition, automated microarray systems that are now commercially available are potentially cheaper and may be more efficient and more accurate than serologic whole allergen specific IgE techniques.[5] Epitope diversity has been demonstrated in prior studies to correlate with reaction severity of patients with food allergies to peanut,[4,9,10] milk,[3,11–13] and egg.[2,14] Thus, we wondered whether identification of the component diversity of an automated microarray system (ISAC; Thermo Fisher Scientific, Waltham, Massachusetts) could be used to clinically diagnose food allergy. In addition, prior studies of microarray-based IgE detection have included individuals with food-exacerbated AD within their food allergic cohort,[15,16] which could cloud results given additional non–IgE-mediated mechanisms in AD.[17,18] Thus, we separated this subset in our analysis. The purpose of our study was to assess the utility of a newly available automated system using microarray technology reporting in

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a semiquantiative determination of units (ISAC). We wanted to see how this corresponded to patient-reported food allergy and food-triggered AD. First, we analyzed odds ratios (ORs) using self-reported food allergy reactions to milk, egg, and peanut as independent variables. Dependent variables included the diversity of detectable components and specific individual detectable components. Second, we looked at how well these variables related to reported milk-, egg-, and peanut-related AD, using the same method.

Methods

Participants in this study were part of a cohort for an ongoing food allergy genetics study for which data have not yet been published. Recruitment occurred during a food allergy informational session presented at a community town hall meeting in Lakewood, New Jersey. This community is composed of 54,500 members of Jewish descent,[19] for which a driving force for migration into this area was the establishment of the Lakewood Yeshiva in 1943.[20] A sign-up sheet was provided for families who reside in the area interested in participating in food allergy research. To be included, participants had to be either a child with reported food allergy and/or food-associated AD or an immediate family member (biological parent or sibling) of this child. Approval was obtained from the Children's Hospital of Philadelphia institutional research board and written informed consent obtained from all research participants. A senior investigator (J.M.S.) concluded that history of food allergy along with skin test and specific IgE when available were consistent with IgE-mediated food allergy. We used established criteria adapted from Thompson and Hanifen[21] (eTable 1). Return visits to Lakewood were scheduled for blood collection for microarray tests (eg, ImmunoCAP and ISAC). Components assessed on the ISAC chip were to milk (Bos d 4, Bos d 5, Bos d 8, Bos d lactoferrin), egg (Gal d 1, Gal d 2, Gal d 3, Gal d 5), and peanut (Ara h 1, Ara h 2, Ara h 3, Ara h 6). Components were considered positive if the result was 0.3 ISAC standardized units (ISU) or more.[22] Summary statistics were performed using STATA12 statistical software (StataCorp, College Station, Texas). $P < .05$ was considered significant. Logistic regression was used to determine whether positivity for individual microarray components or diversity of components significantly increased the odds of having corresponding self-reported food allergy or food-associated AD. For individual components, we calculated sensitivity, specificity, positive predictive value, and negative predictive value. Because there were multiple peanut components of significance, we developed a scoring tool to relate the number of positive peanut components to reported IgE-mediated food allergy. We adapted a previously used scoring system[23] and assigned a score of +3 to highly significant variables ($P < .001$), a score of +2 to those with P values between <.001 and .005, and score of +1 to those with P values between .005 and .05. We then reviewed how well these scores related to reported food symptoms.

Results

Seventy-three patients from 23 families were recruited. Demographic information regarding age, sex, atopy, and adverse reactions to food are listed in Table 1. Of the 20 patients with self-reported milk food allergy, 13 had SPT and/or FEIA above a 95% predictive decision point,[6,24] 3 had positive testing not above this threshold, and 4 had no available records. For the 10 patients with self-reported egg allergy, 7 had SPT and/or FEIA at above the 95% predictive decision point, 2 had positive test results not above this threshold, and 1 did not have available test results. Among the 6 individuals with self-reported peanut allergy, 3 had SPT or FEIA above the 95% positive predictive value threshold, 1 had a positive test result not above this threshold, and 2 did not have available records. Other foods perceived to have caused IgE-mediated symptoms on ingestion were sesame (n = 10), tree nuts (n = 7), fish (n = 2), and soy (n = 1). For reported food-triggered AD, milk, egg, peanut, wheat, and sesame were reported triggers.

Table 1
Demographic characteristics of the study patients[a]

Characteristic	No. (%) of patients (N = 73)
Age, mean (range), y	10 (1-38)
Male	39 (53)
Asthma	16 (22)
Allergic rhinitis	21 (29)
Food allergy	23 (32)
Atopic dermatitis	21 (29)
Specific food allergies	
Milk	20 (27)
Egg	10 (14)
Peanut	6 (8)
Other foods	19 (26)
Food-related atopic dermatitis	
Milk	10 (14)
Egg	7 (10)
Peanut	2 (3)
Other foods	3 (4)

[a]Data are presented as number (percentage) of patients unless otherwise indicated.

Relating ISAC Component Diversity to Self-reported Food Allergy

The odds were significantly higher in patients with greater component diversity for the culprit foods, as indicated in Table 2 (peanut: OR, 10.2; 95% confidence interval [CI], 2.08-49.75; $P < .004$; milk: OR, 38.7; 95% CI, 6.05-247.24; $P < .001$; egg: OR, 3.34; 95% CI, 1.50-7.43; $P < .003$). The odds of reported IgE-mediated peanut allergy were also significant for individually positive Ara h 1, Ara h 2, and Ara h 6, whereas for milk, Bos d 4 was significant.

We then assessed Ara h 1, Ara h 2, and Ara h 6 within the context of the aforementioned scoring tool. The points allotted for each component were based on the P values (Table 1). No component reached $P < .001$. With this tool, 50 of 53 patients who tolerated peanut were correctly identified with a score of zero. The remaining 3 patients each had a score of 3. All 6 patients with reported IgE-mediated reactions to peanut had positive scores. Five of these patients had a score of 4 and 1 had a score of 2.

Table 2
Logistic regression analysis evaluating the association between microarray component outcomes and history of reported IgE-mediated food allergy reaction

Food	OR (95% CI)	*P* value	Score
Peanut			
Ara h 1	1.15 (1.03-1.28)	.02	+1
Ara h 2	1.29 (1.08-1.52)	.003	+2
Ara h 3	…[a]		
Ara h 6	1.16 (1.03-1.31)	.01	+1
No. of positive components	10.16 (2.08-49.75)	.004	
Milk			
Bos d 4	9.21 (1.96-43.4)	.005	
Bos d 5	…		
Bos d 8	…		
Bos d lactoferrin	1.41 (0.25-7.80)	.69	
No. of positive components	38.67 (6.05-247.24)	<.001	
Egg			
Gal d 1	1.17 (0.96-1.41)	.11	
Gal d 2	1.60 (0.86-2.96)	.14	
Gal d 3	1.03 (0.71-1.48)	.89	
Gal d 5	…		
No. of positive components	3.34 (1.50-7.43)	.003	

Abbreviations: CI, confidence interval; OR, odds ratio.
[a]Ellipses indicate inadequate sample size available to perform logistic regression.

Table 3
Sensitivities and Specificities of Microarray Components in Predicting Reported IgE-Mediated Food Allergy

Food	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Peanut				
Ara h 1	100	98	86	100
Ara h 2	83	96	71	98
Ara h 3	33	100	71	98
Ara h 6	83	96	71	88
Milk				
Bos d 4	65	98	93	88
Bos d 5	60	100	100	87
Bos d 8	45	100	100	83
Bos d lactoferrin	5	98	50	73
Egg				
Gal d 1	40	96	67	90
Gal d 2	20	98	67	90
Gal d 3	30	94	50	8
Gal d 5	20	100	100	87

Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

In general, individual components had high specificities but lower sensitivities (Table 3). For peanut, sensitivity and specificity was highest for Ara h 1, at 100% and 98%, respectively. Specificities of individual milk and egg were similarly high (range, 96%-100%). A logistic regression analysis looking at FEIA values for these foods and reported food allergy was not significant in any of these foods (data not shown).

Relating Microarray Component Diversity to Reported Food-Triggered AD

Culprit foods implicated in food-triggered AD included milk (n = 10), egg (n = 7), and peanut (n = 2). Peanut-triggered AD was not analyzed because of low numbers of cases. Results are listed in Table 4. For egg-triggered AD, the number of positive components reached statistical significance as a dependent variable (OR, 2.21; 95% CI, 1.07-4.56; $P = .03$). Individual components did not reach significance. Table 5 lists the calculated sensitivity and specificity of individual components for milk- and egg-associated AD. In general, individual components and combined numbers of components had high specificity for milk (range, 75%-97%) and egg (range, 91%-98%) but had relatively lower sensitivities (0%-38% for milk and 0%-38% for egg).

Discussion

Our results suggest that a high number of food components are a good indicator of food IgE-mediated allergy. Of interest to us, prior studies have identified certain components to be more specific than others for discriminating allergy; however, these components appear to have difference prevalence rates, depending on the population studied. For example, Ara h 1 was detected in more than 90% of peanut-sensitive American patients by Burks et al,[25] whereas Beyer[26] reported 75% prevalence. Clarke et al[27] found 73% prevalence in United Kingdom subjects, and Kleber-Janke et al[28] found 65% prevalence in a German cohort. Vereda et al[29] found immunologic differences among Spanish, Swedish, and US populations, with Ara h 2 being lowest in the Spanish group. Our Lakewood community was a unique, geographically homogeneous cohort in which Ara h 1, Ara h 2, and Ara h 6 were all significantly related to reported food allergy. Our pilot scoring tool incorporating these peanut components was helpful in relating to reported food allergy. In the future, a validated scoring tool incorporating significant components to predict food allergy could significantly affect management. Future studies using oral food challenge as the standard for comparison are therefore very much desired.

Table 4
Logistic Regression Analysis Evaluating the Association Between Milk and Egg Microarray Component Outcomes and History of Reported Food-Triggered Atopic Dermatitis

Food	OR (95% CI)	*P* value
Milk		
Bos d 4	1.04 (0.89-1.22)	.63
Bos d 5	1.02 (0.88-1.17)	.83
Bos d 8	1.06 (0.79-1.42)	.71
Bos d lactoferrin	…[a]	
No. of positive components	1.63 (0.92-2.87)	.09
Egg		
Gal d 1	2.05 (0.56-7.51)	.28
Gal d 2	3.81 (0.45-32.65)	.22
Gal d 3	1.95 (0.82-4.62)	.13
Gal d 5	…	
No. of positive components	2.21 (1.07-4.56)	.03

Abbreviations: CI, confidence interval; OR, odds ratio.
[a]Ellipses indicate inadequate sample size available to perform logistic regression.

Table 5
Sensitivities and Specificities of Milk and Egg Microarray Components in Predicting Reported Food-Triggered Atopic Dermatitis

Food	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Milk				
Bos d 4	38	82	20	91
Bos d 5	38	86	25	92
Bos d 8	34	86	27	86
Bos d lactoferrin	0	97	0	89
Egg				
Gal d 1	38	95	50	91
Gal d 2	33	98	67	93
Gal d 3	25	93	67	90
Gal d 5	0	96	0	87

Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

In our analysis of egg components, Gal d 1 had high specificity (96%) for reported food allergy. Its sensitivity (40%) was higher than other egg components. These results are consistent with other studies, which suggest that Gal d 1 is the immunodominant epitope in persistent egg allergy.[14,30–32] In regard to milk components, Bos d 8 did not have a higher specificity and sensitivity compared with other components. This finding is in contrast to finding from D'Urbano et al[17] that the most commonly detected milk component in children with positive milk oral challenge results was Bos d 8 (46.5%). Because this study was performed in Italy, geographic variation is one variable that may account for this difference.

Few studies to date address the role of microarray testing in diagnosing food-triggered AD. In 20 adult patients with AD, 65% had detectable cross-allergens, such as birch Bet v 1, alder Aln g 1, apple Mal d 1, and celery Api g 1.[33] In pediatric studies, results from patients with food-triggered AD were combined with those from patients with type 1 mediated food allergy. Egger et al[16] reported that Gal d 1 and Gal d 2 were the major detectable components in 25 children with suspected egg allergy, 16 of which had flare of their AD on oral food challenge. Within a cohort of egg and/or milk allergic patients studied for developing microarray clinical decision points for oral food challenge prediction, 8% demonstrated AD exacerbation on double-blinded, placebo-controlled oral food challenge. More recently, Hochwallner et al[34] found that patients who reported skin-only symptoms to milk had lower numbers and amounts of detectable components on microarray compared with those with reported severe systemic reactions. In our study, we found that the number of positive egg components significantly increased the odds of having had reported egg-triggered AD. Further validation of this association and similar relationships for other foods requires larger sample sizes and double-blinded, placebo-controlled food allergy testing.

The ISAC has several potential benefits. It requires a small amount of blood, and many different allergens can be assessed at once, particularly for those with multiple food allergies. Performance-wise, 2 studies reported that microarray testing was comparable to FEIA in predicting milk- and egg-positive oral food challenge results.[15,17] We demonstrated that ISAC microarray results relate well to reported food reactions. Thus, currently ISAC microarray testing may be a helpful adjunct test to SPT and FEIA, which have high sensitivities but low specificities. In the future, with increased diagnostic accuracy of microarray, as reported by Lin et al[9] using bioinformatics methods, we anticipate that microarray testing will become a more valuable adjunctive method of investigating food allergy.

The first limitation to our study is that oral food challenges were not performed to substantiate food allergy histories. Although we could not verify the reported food-related reactions, we identified food allergy cases based on histories with timing and symptoms consistent with such a reaction.[35,36] Second, many patients with reported milk, egg, or peanut food allergy had prior documentation of positive SPT and/or IgE test results to the culprit food. Within the group, 9 patients had records of milk FEIA, 12 had egg FEIA, and 12 had peanut FEIA. Nonetheless, for food-related AD, parent history can lack sensitivity,[8] and thus oral challenges are needed to support our findings for this group. A third limitation is that we studied a population in one geographic setting; thus, results may not be generalizable to other groups. This limitation may explain why Ara h 2 did not have higher specificity compared with other peanut components, as has been found previously in a pediatric population from the United Kingdom.[37,38] Nonetheless, our findings are in keeping with prior studies and suggest that the ISAC immunoassay is a useful additional tool for food allergy diagnosis.[37,39] Future studies should be performed to validate efficacy of the microarray immunoassay in clinical practice.

In conclusion, we report that high diversity of detectable peanut, egg, and milk allergen components relates well to reported allergy reaction to these suspected foods, as does increased total sum of peanut and milk components but to a lesser degree. With proper development of a scoring tool, this serologic testing may become valuable in predicting food allergy.

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Supplementary Data

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eTable 1
Food Allergy and Food-Associated Atopic Dermatitis Questionnaire

1. Do you feel that food contributes to your child's eczema?
2. Has you child ever had an allergic reaction to a food or foods?
   1. If so, which food(s)?
   2. What reaction occurred?
      1. Hives
      2. Lip/face swelling
      3. Swelling/tightening of mouth/throat
      4. Chest tightening, wheezing or cough
      5. Vomiting, diarrhea or cramping
      6. Worsening of eczema (describe)
      7. Other symptoms (describe)
   3. How quickly after eating did the reaction occur?
   4. Has your child had any type of testing for allergy? If so, what test?

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Thymic stromal lymphopoietin–elicited basophil responses promote eosinophilic esophagitis

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Eosinophilic esophagitis (EoE) is a food allergy–associated inflammatory disease characterized by esophageal eosinophilia. Current management strategies for EoE are nonspecific, and thus there is a need to identify specific immunological pathways that could be targeted to treat this disease. EoE is associated with polymorphisms in the gene that encodes thymic stromal lymphopoietin (TSLP), a cytokine that promotes allergic inflammation, but how TSLP might contribute to EoE disease pathogenesis has been unclear. Here, we describe a new mouse model of EoE-like disease that developed independently of IgE, but was dependent on TSLP and basophils, as targeting TSLP or basophils during the sensitization phase limited disease. Notably, therapeutic TSLP neutralization or basophil depletion also ameliorated established EoE-like disease. In human subjects with EoE, we observed elevated *TSLP* expression and exaggerated basophil responses in esophageal biopsies, and a gain-of-function *TSLP* polymorphism was associated with increased basophil responses in patients with EoE. Together, these data suggest that the TSLP-basophil axis contributes to the pathogenesis of EoE and could be therapeutically targeted to treat this disease.

EoE is a food allergy–associated inflammatory disease that affects children and adults[1–3]. In industrialized countries, the incidence of EoE has increased dramatically in the past 30 years, resulting in a considerable public health and economic burden[2,4,5]. EoE is characterized by esophageal eosinophilia and inflammation and histological changes in the esophagus associated with stricture, dysphagia and food impaction[1–3]. Currently, treatment strategies for EoE are nonspecific and impose a burden on patients. Although swallowed topical steroids can be effective in limiting EoE-associated inflammation, there are concerns regarding the long-term use of steroids, particularly in children[2,6]. Adherence to an elemental diet that eliminates exposure to foods that trigger EoE results in resolution of symptoms in many patients; however, this approach requires disruptive changes in lifestyle and eating habits[2,6,7]. Thus, there is a need to identify new drug targets and more specific therapies[7]. The observations that immune suppression or removal of dietary trigger foods can ameliorate EoE symptoms indicate that EoE is a food antigen–driven

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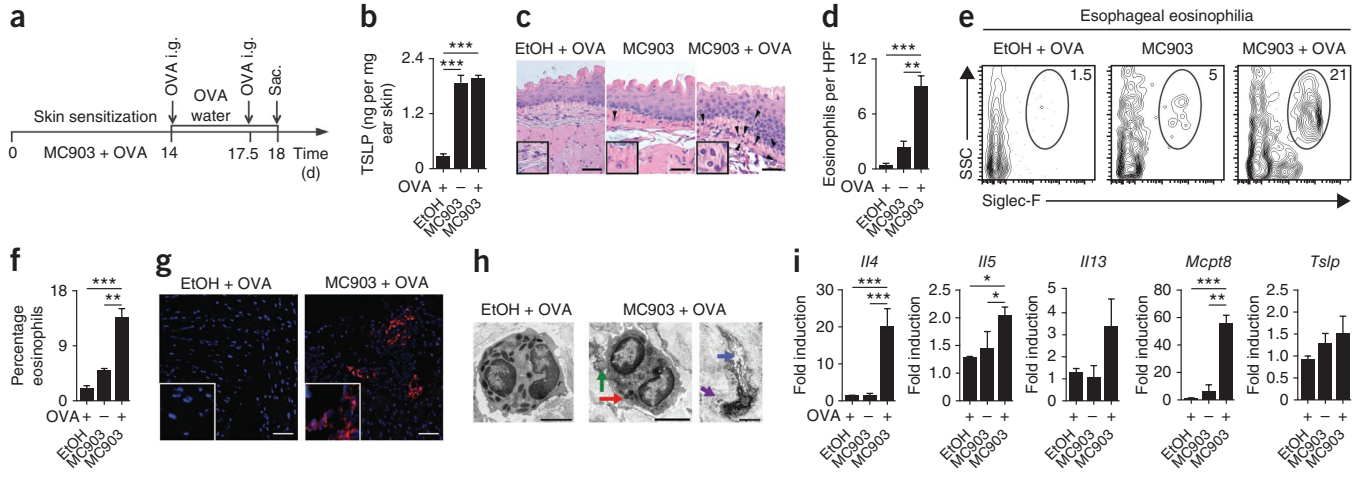
disease mediated by aberrant immune responses[1,2,8]. Therefore, targeting the dysregulated immunological pathways that underlie EoE could offer new treatment strategies for this disease.

Studies investigating the immunological mechanisms that mediate EoE have shown that various immune cell types, including eosinophils, mast cells, type 2 helper T (T_H2) cells that produce interleukin-4 (IL-4), IL-5, and IL-13, and IgE-producing B cells, may contribute to esophageal inflammation during EoE[1–3,9]. Further, recent work has shown that there is a strong association between a gain-of-function polymorphism in the gene that encodes the predominantly epithelial cell–derived cytokine TSLP and the development of EoE in children[10,11]. TSLP is associated with multiple allergic disorders[10–16] and is thought to promote allergic inflammation by activating dendritic cells, inducing T_H2 cell responses, supporting IgE production and eliciting the population expansion of phenotypically and functionally distinct basophils[12,17–21]. However, whether TSLP directly promotes inflammatory responses associated with EoE and the mechanisms by which polymorphisms in *TSLP* and increased TSLP expression may contribute to the pathogenesis of EoE in patients has been unknown.

RESULTS

A new mouse model of experimental EoE-like disease

To investigate whether TSLP directly promotes EoE disease pathogenesis, we developed a new mouse model of EoE-like disease that is associated with exaggerated TSLP production. Multiple studies in mouse models and humans suggest that sensitization to food allergens may occur at sites where the skin barrier is disrupted, such as atopic dermatitis lesions[22–24]. Thus, we employed a model in which mice were epicutaneously sensitized to a food antigen, ovalbumin (OVA), on a developing atopic dermatitis–like skin lesion induced by topical treatment with the vitamin D analog MC903 (**Fig. 1a**). Consistent with previous reports[17,25–27], wild-type (WT) BALB/c mice treated epicutaneously with the vitamin D analog MC903 showed increased TSLP expression in the skin compared to ethanol vehicle–treated control mice (**Fig. 1b**). Epicutaneous sensitization to and subsequent oral challenge with OVA resulted in the development of experimental EoE-like disease that was characterized by inflammation, edema and eosinophilia in the esophagus, as measured histologically and quantified by enumeration of eosinophils per high-power field (HPF) (**Fig. 1c**,**d**). Flow cytometric analysis (**Fig. 1e**,**f**) and immunofluorescence staining (**Fig. 1g**) also demonstrated that there was an accumulation of eosinophils in esophageal tissues of mice with EoE-like disease, and electron microscopic (EM) analysis revealed the presence of degranulated eosinophils in these tissues (**Fig. 1h**). We also observed significantly higher expression of genes that encode T_H2 cytokines and the basophil-specific protease *Mcpt8* and a trend toward increased *Tslp* expression in esophageal tissues of mice with EoE-like disease compared to control mice (**Fig. 1i**). Further, we observed a similar



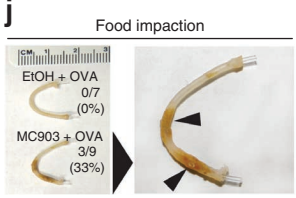


Figure 1 Experimental mouse model of EoE-like disease. (**a**) Schematic of EoE-like disease mouse model in which WT BALB/c mice are epicutaneously sensitized for 14 d with OVA on a developing atopic dermatitis–like skin lesion, challenged intragastrically (i.g.) with OVA on days 14 and 17.5 and sacrificed (sac.) at day 18. (**b**) TSLP (ng per mg of ear skin) expression in supernatants of overnight-cultured skin (ears) measured by ELISA. Data are from one experiment (EtOH + OVA, $n = 3$; MC903, $n = 3$; MC903 + OVA, $n = 4$) and are representative of three independent replicates. EtOH, ethanol. (**c**) Histological sections (H&E staining) from the esophagus. Arrowheads identify tissue-infiltrating eosinophils. Scale bar, 25 µm. Insets: ×4 magnification of whole image focusing on eosinophils. (**d**) Number of eosinophils per HPF in the esophagus. (**e**) Representative flow cytometry plots showing frequencies of eosinophils in esophageal tissues. Data in **c**–**e** are from one experiment (EtOH + OVA, $n = 3$; MC903, $n = 3$; MC903 + OVA, $n = 4$) and are representative of three or more independent replicates. (**f**) Frequencies of eosinophils in esophageal tissues, as measured by flow cytometry. Data are from three pooled experiments (EtOH + OVA, $n = 7$; MC903, $n = 8$; MC903 + OVA, $n = 11$). (**g**) Immunofluorescence staining for eosinophils (Siglec-F–specific mAb, red) in esophageal tissues. Counterstaining with DAPI (blue). Scale bar, 25 µm. Images are representative of two controls and three EoE-like disease samples. Insets: ×4 magnification of whole image focusing on eosinophils. (**h**) Representative EM image of an eosinophil in the esophagus of control mice with intact granules with electron dense cores (left) or degranulating eosinophils in MC903 + OVA–treated mice (right), showing loss of electron density in granule cores (red arrow), granule extrusion channels (blue arrow) and complete loss of granule contents (green arrow) into the extracellular matrix (purple arrow). Scale bar, 2 µm. (**i**) mRNA expression of T_H2 cytokines (*Il4*, *Il5*, *Il13*), the basophil-specific protease *Mcpt8* and *Tslp* in the esophagus. Data depicted are from one experiment (EtOH + OVA, $n = 3$; MC903, $n = 3$; MC903 + OVA, $n = 4$) and are representative of three independent replicates. *y* axis shows fold induction compared to controls (see Online Methods). (**j**) Representative images of esophagi, with incidence of impaction. Arrowheads identify impacted food. Data depicted are from two pooled experiments (EtOH + OVA, $n = 7$, MC903 + OVA, $n = 9$). All parameters were assessed 12 h post-final oral antigen challenge. Data in **a**–**i** are from mice challenged twice with OVA, and data in **j** are from mice challenged six times with OVA. Results are shown as mean ± s.e.m., and a nonparametric, one-way Kruskal-Wallis analysis of variance (ANOVA) with Dunn's *post hoc* testing was used to determine significance. $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$.

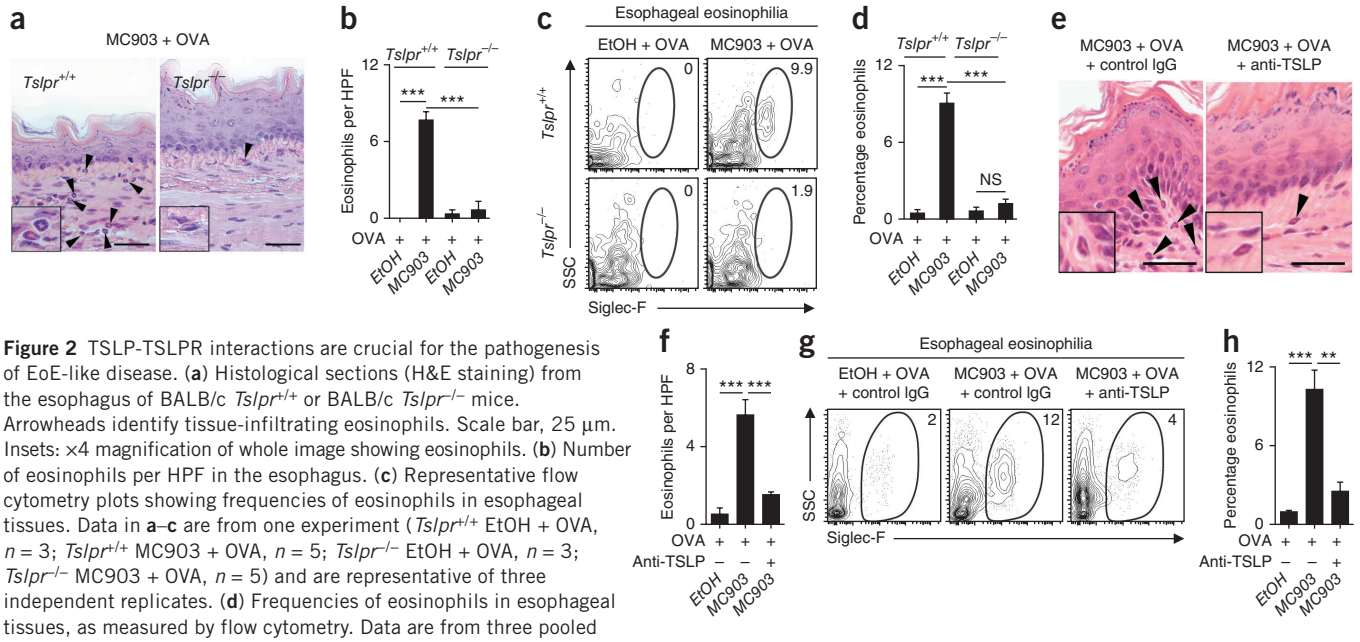


Figure 2 TSLP-TSLPR interactions are crucial for the pathogenesis of EoE-like disease. (**a**) Histological sections (H&E staining) from the esophagus of BALB/c *Tslpr*$^{+/+}$ or BALB/c *Tslpr*$^{-/-}$ mice. Arrowheads identify tissue-infiltrating eosinophils. Scale bar, 25 µm. Insets: ×4 magnification of whole image showing eosinophils. (**b**) Number of eosinophils per HPF in the esophagus. (**c**) Representative flow cytometry plots showing frequencies of eosinophils in esophageal tissues. Data in **a**–**c** are from one experiment (*Tslpr*$^{+/+}$ EtOH + OVA, $n = 3$; *Tslpr*$^{+/+}$ MC903 + OVA, $n = 5$; *Tslpr*$^{-/-}$ EtOH + OVA, $n = 3$; *Tslpr*$^{-/-}$ MC903 + OVA, $n = 5$) and are representative of three independent replicates. (**d**) Frequencies of eosinophils in esophageal tissues, as measured by flow cytometry. Data are from three pooled experiments (*Tslpr*$^{+/+}$ EtOH + OVA, $n = 5$; *Tslpr*$^{+/+}$ MC903 + OVA, $n = 11$; *Tslpr*$^{-/-}$ EtOH + OVA, $n = 5$; *Tslpr*$^{-/-}$ MC903 + OVA, $n = 12$). (**e**) Histological sections (H&E staining) from the esophagus of WT BALB/c mice treated with an isotype control or TSLP-specific mAb (anti-TSLP). Arrowheads identify tissue-infiltrating eosinophils. Scale bar, 50 µm. Insets: ×4 magnification of whole image showing eosinophils. (**f**) Number of eosinophils per HPF in the esophagus. (**g**) Representative flow cytometry plots showing frequencies of eosinophils in esophageal tissues. Data in **e**–**g** are from one experiment (EtOH + OVA + IgG, $n = 3$; MC903 + OVA + IgG, $n = 3$; MC903 + OVA + anti-TSLP mAb, $n = 3$) and are representative of three independent replicates. (**h**) Frequencies of eosinophils in esophageal tissues, as measured by flow cytometry. Data are from three pooled experiments (EtOH + OVA +IgG, $n = 5$; MC903 + OVA + IgG, $n = 9$; MC903 + OVA + anti-TSLP mAb, $n = 10$). All parameters were assessed 12 h after final oral antigen challenge. Data are from mice challenged twice with OVA. Results are shown as mean ± s.e.m., and a nonparametric, one-way Kruskal-Wallis ANOVA with Dunn's *post hoc* testing or a nonparametric, two-way ANOVA with Bonferroni's *post hoc* testing were used to determine significance. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. NS, not significant.

pattern of EoE-like disease in mice that were epicutaneously sensitized to crude peanut extract (CPE) on an atopic dermatitis–like skin lesion (**Supplementary Fig. 1a–c**), confirming that sensitization to a natural food allergen in the presence of elevated amounts of TSLP results in experimental EoE-like disease. Eosinophil accumulation in this model was not restricted to the esophagus, as mice with EoE-like disease also showed eosinophilia in the gastrointestinal tract after epicutaneous sensitization and oral challenge with OVA (**Supplementary Fig. 1d,e**) associated with antigen-specific T_H2 cytokine responses in the mesenteric lymph node and spleen (**Supplementary Fig. 1f,g**).

EoE in humans is diagnosed on the basis of immunological parameters and the presence of physiological changes in esophageal tissue and signs of esophageal dysfunction, including food impaction, which occurs in approximately 40% of patients with EoE[1–3,28]. To assess whether clinical manifestations of EoE were present in the experimental mouse model of EoE-like disease, we challenged mice that had existing EoE-like disease repeatedly with OVA to induce prolonged esophageal inflammation. Although analysis using optical coherence tomography (OCT), which allows for high-resolution imaging of live biological tissues based on optical scattering[29,30], revealed that EoE-like disease was characterized by minimal changes in the thickness of the esophageal epithelium, (**Supplementary Fig. 2a,b**), prolonged esophageal inflammation was associated with food impaction in the esophagus. Approximately 30% of fasted mice with EoE-like disease exhibited food impaction at the time of killing, but we never observed food impaction in the esophagus of control (ethanol)-treated mice (**Fig. 1j**). Collectively, these data indicate that this new model of EoE-like disease is characterized by a number of immunological and pathophysiological changes in esophageal tissues and signs of esophageal dysfunction similar to those observed in humans with EoE[1–3,31–34].

EoE-like disease is dependent on TSLP but independent of IgE

To determine whether TSLP directly promotes the pathogenesis of experimental EoE-like disease in mice, we epicutaneously sensitized WT BALB/c (*Tslpr*$^{+/+}$) mice or mice deficient in the TSLP receptor (TSLPR) (*Tslpr*$^{-/-}$) to OVA followed by oral antigen challenge (see **Fig. 1a**). Whereas sensitized and challenged *Tslpr*$^{+/+}$ mice showed esophageal eosinophilia and associated inflammation, *Tslpr*$^{-/-}$ mice did not develop esophageal eosinophilia (**Fig. 2a–d**). Using an alternative approach to abrogate TSLP signaling, we found that multiple systemic treatments with a monoclonal antibody (mAb) that neutralizes TSLP during epicutaneous sensitization with OVA in WT BALB/c mice also limited eosinophil infiltration in the esophagus after oral challenge (**Fig. 2e–h**).

To test whether TSLP was sufficient for the development of EoE-like disease during epicutaneous sensitization, we intradermally injected mice with exogenous recombinant TSLP (rTSLP) in the presence or absence of OVA and challenged them orally (**Supplementary Fig. 3a**). Mice sensitized to OVA in the presence of rTSLP also showed esophageal eosinophilia after oral challenge compared to mice treated with OVA alone or rTSLP alone (**Supplementary Fig. 3b**). In a complementary approach, *Tslpr*$^{+/+}$ mice were treated with control antibody or a TSLP-specific mAb, and *Tslpr*$^{-/-}$ mice were sensitized with OVA on tape-stripped skin (**Supplementary Fig. 3c**). Tape-stripping was associated with elevated local TSLP production following physical

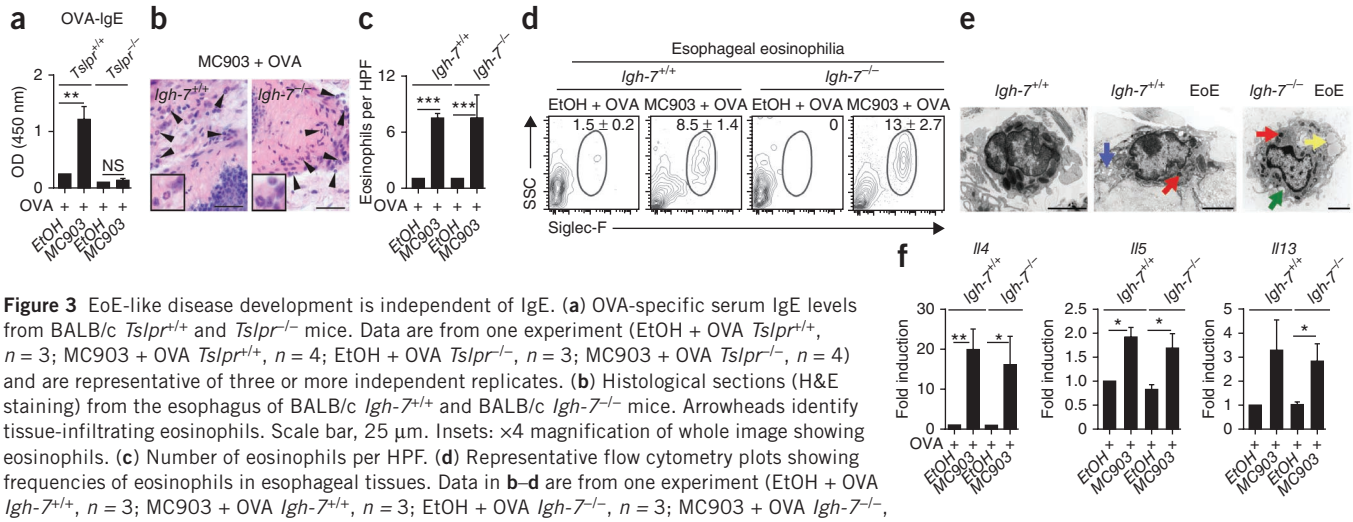


Figure 3 EoE-like disease development is independent of IgE. (**a**) OVA-specific serum IgE levels from BALB/c *Tslpr*$^{+/+}$ and *Tslpr*$^{-/-}$ mice. Data are from one experiment (EtOH + OVA *Tslpr*$^{+/+}$, $n = 3$; MC903 + OVA *Tslpr*$^{+/+}$, $n = 4$; EtOH + OVA *Tslpr*$^{-/-}$, $n = 3$; MC903 + OVA *Tslpr*$^{-/-}$, $n = 4$) and are representative of three or more independent replicates. (**b**) Histological sections (H&E staining) from the esophagus of BALB/c *Igh-7*$^{+/+}$ and BALB/c *Igh-7*$^{-/-}$ mice. Arrowheads identify tissue-infiltrating eosinophils. Scale bar, 25 µm. Insets: ×4 magnification of whole image showing eosinophils. (**c**) Number of eosinophils per HPF. (**d**) Representative flow cytometry plots showing frequencies of eosinophils in esophageal tissues. Data in **b**–**d** are from one experiment (EtOH + OVA *Igh-7*$^{+/+}$, $n = 3$; MC903 + OVA *Igh-7*$^{+/+}$, $n = 3$; EtOH + OVA *Igh-7*$^{-/-}$, $n = 3$; MC903 + OVA *Igh-7*$^{-/-}$, $n = 4$) and are representative of three or more independent replicates. (**e**) Representative EM image of an eosinophil in the esophagus of control *Igh-7*$^{+/+}$ mice with intact granules with electron-dense cores (left) or degranulating eosinophils in MC903 + OVA treated *Igh-7*$^{+/+}$ (middle) or *Igh-7*$^{-/-}$ (right) mice in various stages of degranulation, with loss of electron density in granule cores (red arrows), formation of granule extrusion channels (blue arrow), complete loss of granule contents (green arrow) and formation of lipid vesicles (yellow arrow). Scale bar, 2 µm. (**f**) mRNA expression of T_H2 cytokines in the esophagus. Data are from one experiment (EtOH + OVA *Igh-7*$^{+/+}$, $n = 3$; MC903 + OVA *Igh-7*$^{+/+}$, $n = 3$; EtOH + OVA *Igh-7*$^{-/-}$, $n = 3$; MC903 + OVA *Igh-7*$^{-/-}$, $n = 3$) and are representative of two independent replicates. *y* axis shows fold induction compared to controls (see Online Methods). All parameters were assessed 12 h after final oral antigen challenge. Data are from mice challenged twice with OVA. Results are shown as mean ± s.e.m., and a nonparametric, two-way ANOVA with Bonferroni's *post hoc* testing was used to determine significance. $^*P \le 0.05$, $^{**}P \le 0.01$; $^{***}P \le 0.001$. NS, not significant.

perturbation of the skin barrier (**Supplementary Fig. 3d** and ref. 35). Whereas *Tslpr*$^{+/+}$ mice treated with control antibody that were sensitized to OVA on tape-stripped skin showed esophageal eosinophilia after oral antigen challenge, *Tslpr*$^{+/+}$ mice treated with a TSLP-specific mAb and *Tslpr*$^{-/-}$ mice did not develop esophageal eosinophilia (**Supplementary Fig. 3e**,**f**). Finally, we assessed the contribution of TSLP to the development of clinical signs of EoE-like disease. Repeated challenge with OVA following sensitization in the presence of MC903 was not associated with changes in the thickness of the esophageal epithelium. However, prolonged esophageal inflammation was associated with an increased incidence of food impaction in the esophagus in *Tslpr*$^{+/+}$ but not *Tslpr*$^{-/-}$ mice (**Supplementary Fig. 4a**,**b**). Collectively, these data indicate that TSLP-TSLPR interactions are necessary and sufficient for the development of experimental EoE-like disease in mice.

TSLP-TSLPR interactions are known to promote the production of IgE[36,37], a key mediator of allergic inflammation[38], and class-switched B cells have been observed in the esophagus of patients with EoE[9,39,40]. In addition, MC903-induced TSLP expression was associated with high amounts of systemic OVA-specific IgE (**Fig. 3a**), suggesting that TSLP-dependent EoE-like disease in mice might be IgE dependent. To directly test this, we epicutaneously sensitized IgE-sufficient WT BALB/c (*Igh-7*$^{+/+}$) mice and IgE-deficient (*Igh-7*$^{-/-}$) mice to OVA in the presence of MC903. Following oral challenge with antigen, both *Igh-7*$^{+/+}$ and *Igh-7*$^{-/-}$ mice showed equivalent EoE-like disease, characterized by esophageal inflammation, elevated tissue eosinophilia (**Fig. 3b**–**d**), the presence of degranulated eosinophils in the esophagus (**Fig. 3e**) and significant increases in gene expression of T_H2 cytokines in esophageal tissues (**Fig. 3f**). These data demonstrate that EoE-like disease can occur in an IgE-independent manner and are consistent with recent findings from clinical studies suggesting that treatment with an IgE-specific mAb does not ameliorate EoE in most patients[41–44]. Together, these data indicate that manipulation of the IgE pathway may not be an effective therapeutic approach for the treatment of EoE.

EoE-like disease depends on basophils

In addition to its role in promoting B cell and IgE responses, TSLP expression is associated with the selective expansion of a distinct population of basophils[17,18]. These data suggest that basophils may contribute to TSLP-dependent, IgE-independent EoE-like disease in mice. Consistent with this hypothesis, MC903-induced expression of TSLP in the skin was associated with TSLP-dependent, IgE-independent systemic basophil responses (**Supplementary Fig. 5a**,**b**). To assess whether basophils contribute to the development of experimental EoE-like disease, we employed an established genetic approach to deplete basophils *in vivo*. C57BL/6 mice in which the diphtheria toxin receptor (DTR) is exclusively expressed by basophils (Baso-DTR$^+$ mice)[17,19,45] and DTR-negative littermate controls (Baso-DTR$^-$ mice) were epicutaneously sensitized and orally challenged with OVA while being treated with diphtheria toxin (**Fig. 4a**). Consistent with results observed in BALB/c mice (**Fig. 1b**), we observed increased local and systemic TSLP production in C57BL/6 Baso-DTR$^-$ and Baso-DTR$^+$ mice sensitized to OVA in the context of MC903 treatment (data not shown). Notably, whereas Baso-DTR$^-$ mice that were epicutaneously sensitized and orally challenged with OVA showed high frequencies of eosinophils in the esophagus, depletion of basophils in Baso-DTR$^+$ mice (**Supplementary Fig. 5c**) led to a reduction in esophageal eosinophilia (**Fig. 4b**–**e**) and a reduction in expression of genes related to T_H2 cytokine responses (**Supplementary Fig. 6a**–**c**).

Using an alternative approach, we treated epicutaneously sensitized and orally challenged WT BALB/c mice with a mAb specific for CD200R3 (Ba103) to deplete basophils[46] (**Fig. 4f**). Mice in which basophils were depleted during sensitization (**Supplementary Fig. 5d**) showed a reduced accumulation of eosinophils in the esophagus compared to control mAb–treated mice after oral challenge with OVA (**Fig. 4g**–**j**). Collectively, these

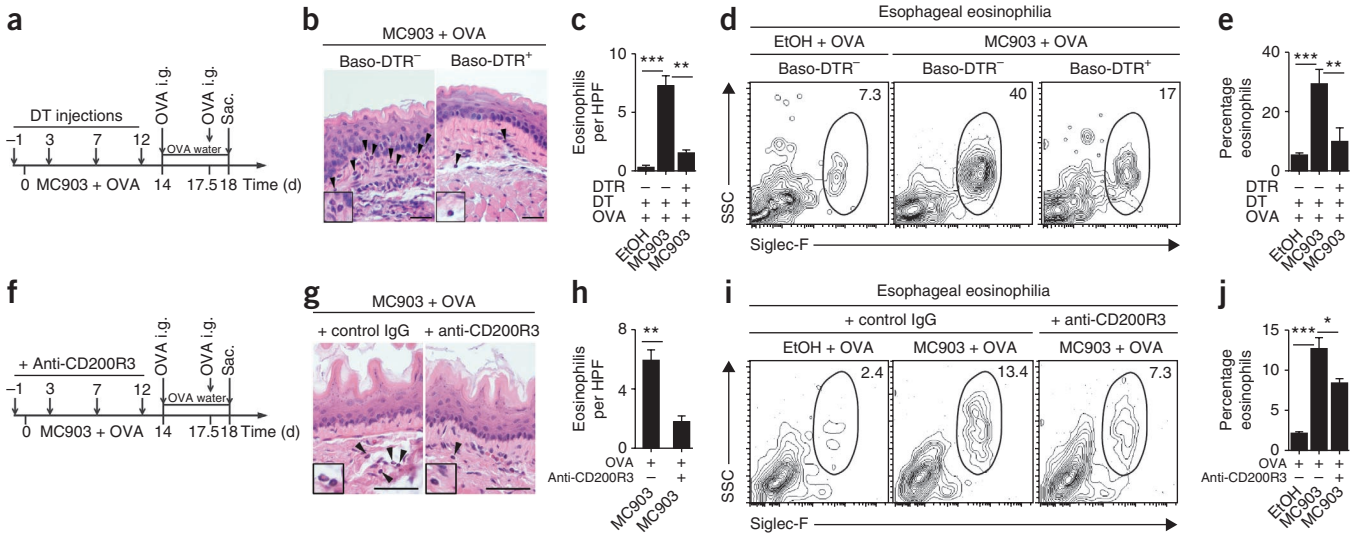


Figure 4 Basophils promote EoE-like disease. (**a**) Schematic of *in vivo* basophil depletion strategy. C57BL/6 (Baso-DTR$^-$) or Baso-DTR$^+$ mice were treated with diphtheria toxin (DT) during the course of epicutaneous sensitization. (**b**) Histological sections (H&E staining) from the esophagus. Arrowheads identify tissue-infiltrating eosinophils. Scale bar, 25 μm. Insets: ×4 magnification of whole image showing eosinophils. (**c**) Number of eosinophils per HPF in the esophagus. (**d**) Representative flow cytometry plots showing frequencies of eosinophils in esophageal tissues. Data in **b**–**d** are from one experiment (Baso-DTR$^-$ EtOH + OVA, $n = 3$; Baso-DTR$^-$ MC903 + OVA, $n = 3$; Baso-DTR$^+$ MC903 + OVA, $n = 4$) and are representative of three independent replicates. (**e**) Frequencies of eosinophils in esophageal tissues, as measured by flow cytometry. Data depicted are from three pooled experiments (Baso-DTR$^-$ EtOH + OVA, $n = 7$; Baso-DTR$^-$ MC903 + OVA, $n = 10$; Baso-DTR$^+$ MC903 + OVA, $n = 11$). (**f**) Schematic of *in vivo* basophil depletion strategy using CD200R3-specific mAb (anti-CD200R3) in WT BALB/c mice. (**g**) Histological sections (H&E staining) from the esophagus. Arrowheads identify tissue-infiltrating eosinophils. Scale bar, 50 μm. Insets: ×4 magnification of whole image showing eosinophils. (**h**) Number of eosinophils per HPF in the esophagus. (**i**) Representative flow cytometry plots showing frequencies of eosinophils in esophageal tissues. Data in **g**–**i** are from one experiment (EtOH + OVA + IgG, $n = 3$; MC903 + OVA + IgG, $n = 3$; MC903 + OVA + anti-CD200R3 mAb, $n = 4$) and are representative of three independent replicates. (**j**) Frequencies of eosinophils in esophageal tissues, as measured by flow cytometry. Data are from three pooled experiments (EtOH + OVA + IgG, $n = 8$; MC903 + OVA + IgG, $n = 9$; MC903 + OVA + anti-CD200R3 mAb, $n = 10$). All parameters were assessed 12 h after final oral antigen challenge. Data are from mice challenged twice with OVA. Results are shown as mean ± s.e.m., and a nonparametric, two-tailed Mann-Whitney *t*-test or a nonparametric, one-way Kruskal-Wallis ANOVA with Dunn's *post hoc* testing were used to determine significance. $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$.

results indicate that basophils are major contributors to the pathogenesis of experimental EoE-like disease in mice and may represent a new therapeutic target to treat this disease in patients.

TSLP or basophils can be targeted to treat EoE-like disease

As TSLP and basophils were required during sensitization for the development of EoE-like disease in mice, we next tested whether the TSLP-basophil pathway could be therapeutically targeted to treat established EoE-like disease. First, we sensitized and challenged mice with OVA to establish EoE-like disease and then treated them systemically with either an isotype control or a neutralizing TSLP-specific mAb during repeated antigen challenge (**Fig. 5a**). Whereas mice with established EoE-like disease treated with a control antibody showed esophageal eosinophilia, mice that were treated with a TSLP-specific mAb had decreased esophageal eosinophilia, as measured histologically (**Fig. 5b**). Flow cytometric analysis also revealed that the total immune cell infiltrate and esophageal eosinophilia were significantly reduced in mice treated with a TSLP-specific mAb compared to mice treated with a control mAb (**Fig. 5c**,**d**).

To test whether basophils contributed to the maintenance of EoE-like disease, we treated mice with established EoE-like disease with an isotype control or basophil-depleting CD200R3-specific mAb during repeated OVA challenge (**Fig. 5e**). Similar to the results observed after neutralization of TSLP, specific depletion of basophils resulted in decreased esophageal eosinophilia, as measured histologically (**Fig. 5f**), and flow cytometric analysis showed a reduction in total immune cell infiltrate and eosinophil numbers in the esophagus (**Fig. 5g**,**h**). To test whether neutralization of TSLP or depletion of basophils was also associated with a resolution of signs of esophageal dysfunction, we treated mice with established EoE-like disease with a control antibody, TSLP-specific mAb, or CD200R3-specific mAb and assessed them for the incidence of food impaction. Whereas we observed food impaction in about 30% of mice treated with a control antibody, we did not observe food impaction in mice in which TSLP or basophil responses were blocked (**Fig. 5i**). Taken together, these data demonstrate that TSLP neutralization or basophil depletion can be used to ameliorate inflammation and clinical symptoms of established experimental EoE-like disease in mice.

The TSLP-basophil axis is associated with EoE in humans

The roles of TSLP and basophils in experimental EoE-like disease in mice (**Figs. 2** and **4**) and the established association between a gain-of-function polymorphism in *TSLP* and EoE in human pediatric subjects[10,11] prompted us to hypothesize that the TSLP-basophil pathway may contribute to the pathogenesis of EoE in humans. To assess whether the TSLP-basophil axis is active in human subjects with EoE, we examined TSLP expression and basophil responses in esophageal biopsies from a cohort of pediatric subjects. We stratified this patient population on the basis of the number of eosinophils counted in histologic sections from esophageal biopsies into the following groups: (i) control subjects without EoE, (ii) subjects with active EoE (≥15 eosinophils per HPF) and (iii) subjects with inactive EoE (<15 eosinophils per HPF and a prior clinical history of active EoE) (**Fig. 6a**). In agreement with previous studies[10,11],

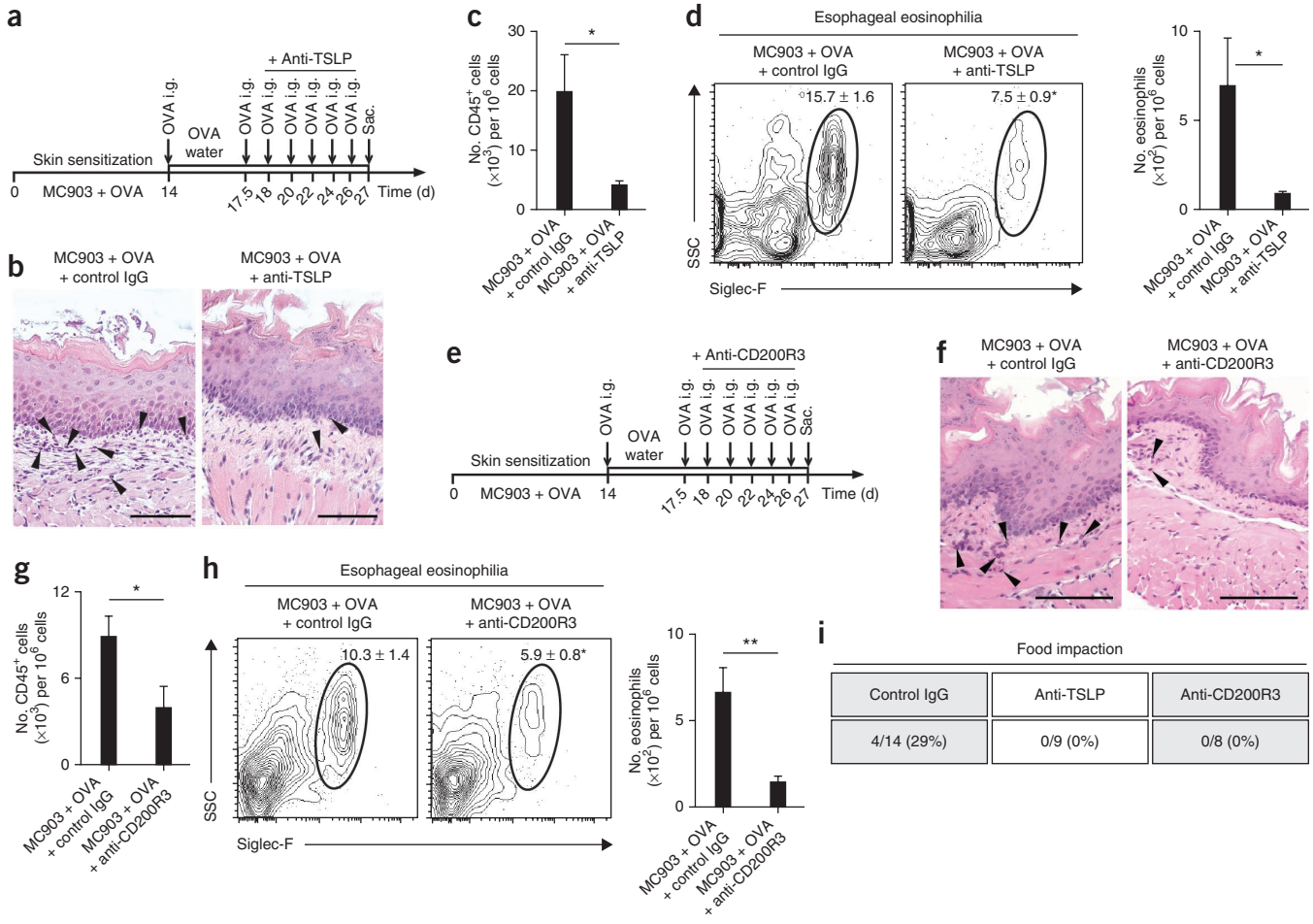


Figure 5 Neutralization of TSLP or depletion of basophils ameliorates established EoE-like disease. (**a**) Schematic of treatment with TSLP-specific mAb in WT BALB/c mice with established EoE-like disease. (**b**) Histological sections (H&E staining) from the esophagus. Arrowheads identify tissue-infiltrating eosinophils. Scale bar, 50 µm. (**c**) Frequencies of CD45$^+$ cells in esophageal tissues, as measured by flow cytometry. (**d**) Representative flow cytometry plots showing frequencies and total numbers of eosinophils in esophageal tissues. Data in **b**–**d** are from one experiment (MC903 + OVA + IgG, $n = 5$; MC903 + OVA + anti-TSLP mAb, $n = 5$) and are representative of three independent replicates. (**e**) Schematic of CD200R3-specific mAb basophil-depletion strategy in WT BALB/c mice in established EoE-like disease. (**f**) Histological sections (H&E staining) from the esophagus. Arrowheads identify tissue-infiltrating eosinophils. Scale bar, 50 µm. (**g**) Frequencies of CD45$^+$ cells in esophageal tissues, as measured by flow cytometry. (**h**) Representative flow cytometry plots showing frequencies and total numbers of eosinophils in esophageal tissues. Data in **f**–**h** are from one experiment (MC903 + OVA + IgG, $n = 4$; MC903 + OVA + anti-CD200R3 mAb, $n = 5$) and are representative of three independent replicates. (**i**) Quantified incidence of food impaction. All parameters were assessed 12 h after final oral antigen challenge. Data are from mice challenged repeatedly with OVA. Results are shown as mean ± s.e.m., and a nonparametric, two-tailed Mann-Whitney *t*-test was used to determine significance. $^*P \leq 0.05$; $^{**}P \leq 0.01$.

TSLP expression in esophageal biopsies was higher in subjects with active EoE compared to control subjects or subjects with inactive EoE (**Fig. 6b**). Immunohistochemical staining revealed that stratified squamous epithelial cells showed positive staining for TSLP in esophageal biopsies from subjects with active EoE (**Fig. 6c**). We then used flow cytometric analysis to identify and quantify the inflammatory cell infiltrate in biopsies. Notably, we observed higher frequencies of cells with a phenotype consistent with that of basophils (lin$^-$CD49b$^+$FcεRI$^+$c-kit$^-$2D7$^+$) in esophageal biopsies from subjects with active EoE compared to those from control subjects or subjects with inactive EoE (**Fig. 6d**,**e**). Further, the frequency of basophils positively correlated (Spearman $r = 0.6638$) with the number of eosinophils counted per HPF in histological sections of esophageal biopsies (**Fig. 6f**). Additionally, we were able to stratify a cohort of adult subjects on the basis of the number of eosinophils counted in histologic sections (**Supplementary Fig. 7a**). Consistent with results observed in pediatric subjects (**Fig. 6d**–**f**), adult subjects with active EoE had a higher (although not statistically significant) frequency of basophils in the esophageal biopsy, as measured using flow cytometry, that positively correlated (Spearman $r = 0.5282$) with the number of eosinophils counted per HPF in histological sections (**Supplementary Fig. 7b**,**c**). Collectively, these data indicate for the first time, to our knowledge, that the TSLP-basophil axis is associated with active EoE in pediatric and adult subjects.

These findings, coupled with the association between the development of EoE and a previously identified gain-of-function polymorphism in *TSLP* associated with *TSLP* overexpression (*TSLP*^{risk})[10], suggested that there may be an association between the *TSLP*^{risk} polymorphism and enhanced basophil responses in human subjects with EoE. To directly test this, we assessed a separate cohort of pediatric subjects with active or inactive EoE genotyped for the presence of the *TSLP*^{risk} polymorphism for basophil frequencies among peripheral blood mononuclear cells (PBMCs). Subjects who were homozygous or heterozygous for the *TSLP*^{risk} polymorphism had significantly higher

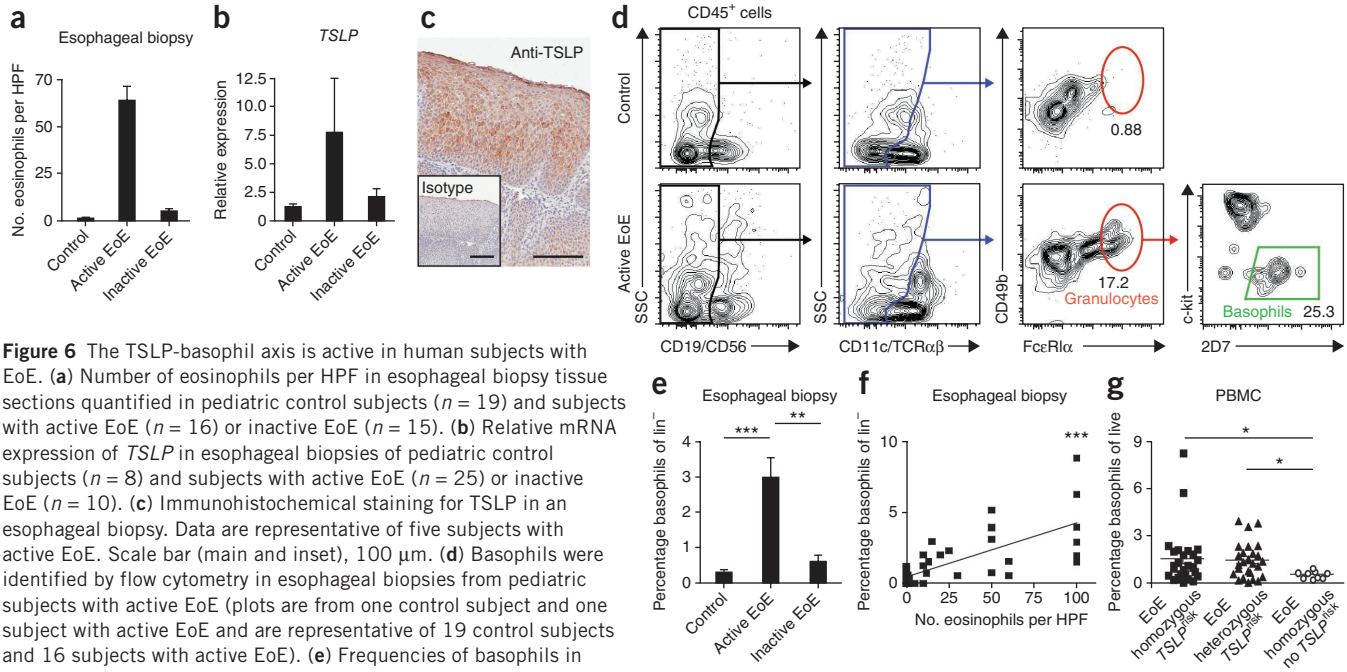


Figure 6 The TSLP-basophil axis is active in human subjects with EoE. (**a**) Number of eosinophils per HPF in esophageal biopsy tissue sections quantified in pediatric control subjects ($n = 19$) and subjects with active EoE ($n = 16$) or inactive EoE ($n = 15$). (**b**) Relative mRNA expression of *TSLP* in esophageal biopsies of pediatric control subjects ($n = 8$) and subjects with active EoE ($n = 25$) or inactive EoE ($n = 10$). (**c**) Immunohistochemical staining for TSLP in an esophageal biopsy. Data are representative of five subjects with active EoE. Scale bar (main and inset), 100 µm. (**d**) Basophils were identified by flow cytometry in esophageal biopsies from pediatric subjects with active EoE (plots are from one control subject and one subject with active EoE and are representative of 19 control subjects and 16 subjects with active EoE). (**e**) Frequencies of basophils in the lin$^-$ compartment (see Online Methods) in esophageal biopsies from pediatric control subjects ($n = 19$) and subjects with active EoE ($n = 16$) or inactive EoE ($n = 15$). (**f**) Correlation of frequencies of basophils in pediatric esophageal biopsies and the number of eosinophils per HPF observed histologically ($n = 50$) (Spearman $r = 0.6638$). (**g**) Frequencies of basophils in the PBMCs of pediatric subjects with EoE who were homozygous ($n = 26$) or heterozygous for the *TSLP*^{risk} polymorphism ($n = 26$) or who lacked the *TSLP*^{risk} polymorphism ($n = 9$), as identified by flow cytometry. All data are shown as mean ± s.e.m., and a nonparametric, two-tailed Mann-Whitney *t*-test or a nonparametric, one-way Kruskal-Wallis ANOVA with Dunn's *post hoc* testing were used to determine significance. Correlation analysis was performed using a nonparametric Spearman correlation (sensitivity analyses were performed), and a linear regression of the data is shown. $^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$.

basophil frequencies in their PBMCs than subjects with EoE who did not carry the *TSLP*^{risk} polymorphism (**Fig. 6g**), which suggests a genetic link between a gain-of-function *TSLP* polymorphism, increased peripheral basophil responses and EoE. As with most human inflammatory diseases such as asthma, inflammatory bowel disease and multiple sclerosis[47–50], the development of EoE probably involves a complex interplay of genetic and environmental factors. However, these data suggest a model in which patients that carry the *TSLP*^{risk} polymorphism have a predisposition toward *TSLP* overexpression and associated peripheral basophilia that may increase the likelihood of developing EoE after encounter with trigger antigens (**Supplementary Fig. 8**).

DISCUSSION

Here we describe a new mouse model in which epicutaneous sensitization to a model food antigen followed by oral antigen challenge results in EoE-like disease. We demonstrate that TSLP and basophils, but not IgE, are required for the development of experimental EoE-like disease in mice and that antibody-mediated neutralization of TSLP or depletion of basophils is effective in preventing the development of experimental EoE-like disease. Targeting TSLP or basophils was also effective in treating established EoE-like disease in mice. In addition, we identify for the first time the presence of enhanced basophil responses in the esophageal biopsy tissue of human subjects with EoE and a genetic link between a gain-of-function polymorphism in *TSLP* and increased peripheral basophil responses.

Although all experimental model systems have limitations and do not recapitulate the diversity of symptoms reported in humans, the model of EoE-like disease we report here is associated with several characteristics of EoE in humans, including esophageal eosinophilia and associated esophageal dysfunction. In addition, this model is also characterized by gastrointestinal eosinophilia and systemic T_H2 cytokine responses. EoE in humans is defined as a disease associated with eosinophilia in the esophagus. However, patients with EoE often suffer from coexisting allergic disorders such as atopic dermatitis, allergic rhinitis, asthma or intestinal food allergy[2,7,51]. These observations suggest that a subset of individuals with EoE with coexisting allergic diseases may present with manifestations of allergic disease at tissue sites outside of the esophagus[52]. Thus, the mouse model of EoE-like disease we describe may recapitulate a pan-allergic disease state present in some humans who have EoE and suffer from additional allergic diseases. Although EoE-like disease in this model develops independently of IgE and is dependent on TSLP and basophils, further studies will be required to investigate whether the gastrointestinal eosinophilia in this model is dependent on IgE or TSLP-elicited basophils.

Previous studies in mouse models and humans have identified various immunological factors that are associated with EoE[1–3,31–34,53–58]. However, recent clinical trials that have targeted some of these factors, including IgE and IL-5, have failed to ameliorate symptoms of disease[2,41,42,44,59,60], suggesting that these factors may not be essential for the pathogenesis of EoE. The demonstration that EoE-like disease in mice can develop independently of IgE but is dependent on TSLP and basophils may explain why previous clinical trials employing other candidate biologic therapies have not been successful. The identification of a role for TSLP and basophils in experimental EoE-like disease in mice, coupled with the association between TSLP and basophil responses and EoE in humans, indicate

that targeting the TSLP-basophil axis may offer new opportunities for the clinical management of EoE in patients.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.N., E.D.T.W., B.S.K., M.C.S., P.R.G., M.G.N., A.B.M., A.A., C.Z. and D.A. designed and performed experiments. A.J.B., K.R.R., P.M.-K., A.C., G.W.F., M.-L.W. and J.M.S. obtained human pediatric and adult esophageal biopsies and peripheral blood samples, K.R.C. analyzed pediatric esophageal biopsy histology, and D.A.H. and T.B.-W. coordinated patient care and clinical studies. A.E.M. and Q.J.S. provided CPE, M.K. provided Baso-DTR mice, K.O.-N. and H.K. provided CD200R3-specific mAb, M.R.C. provided TSLPR-deficient mice and TSLP reagents, J.H.Y. and R.d.W.M. performed staining for human TSLP, and P.M.S. and H.H. provided genotype information on pediatric patients with EoE. M.N., E.D.T.W., B.S.K., M.C.S., P.R.G., A.A., C.Z., M.-L.W., J.M.S. and D.A. analyzed the data. M.N., E.D.T.W., M.C.S. and D.A. wrote the manuscript, and all authors critically reviewed the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mice. Male and female BALB/c and C57BL/6 mice were purchased from the Jackson Laboratories. BALB/c *Tslpr*$^{+/+}$ and BALB/c *Tslpr*$^{-/-}$ mice were provided by Amgen, through Charles River Laboratories. BALB/c *Igh-7*$^{-/-}$ mice and C57BL/6 Baso–DTR mice were bred at the University of Pennsylvania. All mice were used at 8–12 weeks of age, and all experiments employed age-, gender- and genetic strain-matched controls to account for any variations in data sets compared across experiments. Mice were bred and housed in specific pathogen–free conditions at the University of Pennsylvania. Mice requiring medical attention were provided with appropriate veterinary care by a licensed veterinarian and were excluded from the experiments described. No other exclusion criteria existed. All experiments were performed under the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) approved protocols and in accordance with its guidelines.

Reagents and treatments. Mice were treated daily with 2 nmol MC903 (calcipotriol, Tocris Bioscience) in 20 µl of 100% EtOH applied to the ears in the presence of 100 µg OVA for 14 d. As a vehicle control, the same volume of EtOH and OVA was applied. For tape-stripping, mice were shaved on the back, tape-stripped six times with scotch sealing tape and sensitized with 100 µg OVA or saline as control daily for 14 d. For TSLP injections, mice were subcutaneously injected with 5 µg rTSLP in the presence of 100 µg OVA on days 0, 3, 6, 9 and 12. For controls, mice were injected subcutaneously with PBS or rTSLP alone. For CPE sensitization, CPE was made from whole roasted peanuts (Sainsbury's Ltd.) sterilized by gamma irradiation (Lillico Biotech) that were ground in an airflow cabinet using a mortar and pestle. The resulting paste was solubilized in pH 7.4 PBS (Gibco) and sonicated for two 20-min periods, with mixing in between. The solution was then filtered through a 75-µm tissue filter (BD Biosciences) to remove large particles of debris. Lipopolysaccharide content was tested (Lonza) and reported less than 0.006 ng mL^{-1}. Mice were treated daily with 2 nmol MC903 in 20 µl of 100% EtOH on ears in the presence of 100 µg CPE for 14 d. As a vehicle control, the same volume of EtOH and CPE was applied. Mice were challenged i.g. with 50 mg OVA or 10 mg CPE on days 14 and 17.5 and killed on day 18. Upon first i.g. OVA or CPE challenge, mice were continuously fed water containing 1.5 g L^{-1} OVA or given continuous access to whole roasted peanut. Mice subjected to repeated challenge with OVA to induce prolonged inflammation in the esophagus were challenged i.g. with 50 mg OVA on days 14, 17.5, 18, 20, 22, 24 and 26 and killed on day 27. For depletion with TSLP-specific mAb[17], mice were injected with 500 µg of control IgG or TSLP-specific mAb commercially produced by Amgen intraperitoneally every 3 d during the course of the experiment starting at day −1 or every other day starting at day 18. For basophil depletion by diphtheria toxin treatment, Baso-DTR^{+} or Baso-DTR^{-} littermate control mice were treated with 500 ng diphtheria toxin (Sigma) intraperitoneally on days −1, 3, 7 and 12. For depletion with CD200R3-specific mAb (Ba103)[46], mice were injected with 100 µg of control IgG or CD200R3-specific mAb (clone Ba103, provided by H. Karasuyama) intravenously every 4 d during the course of the experiment starting at day −1 or every other day starting at day 18. To assess food impaction in the esophagus, mice exposed to prolonged esophageal inflammation were fasted for at least 30 min and up to 2 h. Mice were then killed, and their esophagi were examined for the presence of impacted food.

Cohort of human subjects with eosinophilic esophagitis. Pediatric participants from a cohort of control subjects or subjects with EoE at the University of Pennsylvania Penn-Children's Hospital of Philadelphia (CHOP) Joint Center for Digestive, Liver and Pancreatic Medicine or the Center for Pediatric Eosinophilic Disorders at CHOP were analyzed and were provided under a CHOP IRB to M.-L.W. and A.J.B. Adult participants from a cohort of control subjects or subjects with EoE being treated at the Hospital of the University of Pennsylvania Division of Gastroenterology were also assessed and were provided under a University of Pennsylvania IRB to G.W.F. and P.M.-K. Written consent was obtained from all participants or their parents or legal guardians, and for pediatric participants, verbal assent from the child was additionally obtained. Subjects defined as having EoE had no other chronic condition except asthma, allergic rhinitis, food allergy, urticaria or atopic dermatitis. Control subjects presented with epigastric abdominal pain but had normal endoscopic and microscopic results. Pediatric subjects with EoE were on proton pump inhibitor therapy, but subjects on systemic corticosteroid treatment or antibiotics were excluded. Subjects with active EoE had an esophageal eosinophil count of ≥15 per HPF after 8 weeks of treatment with a proton pump inhibitor. Subjects with inactive EoE had previously been diagnosed with active EoE but had an esophageal eosinophil count of < 15 per HPF at the time of sample collection. During routine endoscopy, three esophageal biopsies were collected for histological analysis of esophageal eosinophil counts. During the same procedure, two esophageal tissue biopsies were collected for research purposes, for either real-time PCR, immunohistochemistry or flow cytometry. For flow cytometry, single-cell suspensions were made by filtering the mechanically disrupted tissue through a 70-µm filter (BD Biosciences) for flow cytometry. Peripheral blood from pediatric subjects from a cohort of control subjects or subjects with active or inactive EoE that were genotyped for a gain-of-function *TSLP* polymorphism at the University of Pennsylvania Penn-CHOP Joint Center for Digestive, Liver and Pancreatic Medicine or the Center for Pediatric Eosinophilic Disorders at CHOP was analyzed and was provided under a CHOP IRB to J.M.S. and K.R.R. Written consent was obtained from all participants or their parents or legal guardians, and for pediatric participants, verbal assent from the child was additionally obtained. Peripheral blood was collected by venipuncture, and serum was isolated. PBMCs were isolated by Ficoll gradient as previously described[17], and cells were analyzed by flow cytometry. For genotyping of pediatric subjects with EoE, all samples were genotyped on either the Illumina HumanHap 550 or 610 BeadChips according to the manufacturer's protocols. Data normalization and canonical genotype clustering were carried out using the Illumina Genome Studio package. Samples with call rate <98% were excluded from further analysis.

Human real-time PCR and immunohistochemistry. For real-time PCR analysis of gene expression in human esophageal biopsies, human subject biopsy samples were collected and placed in RNA*later* (Ambion). RNA was isolated using the mirVana miRNA Isolation Kit according to the manufacturer's recommendations (Ambion) and reverse transcribed using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems). Quantitative real-time PCR was performed using the Taqman Fast Universal PCR Master Mix kit and preformulated TaqMan Gene Expression Assays for *TSLP* (Applied Biosystems). Reactions were performed in triplicate using 96-well optical plates on a StepOnePlus Real-Time PCR System (Applied Biosystems). GAPDH was used as an endogenous control to normalize the samples using the C_T method of relative quantification, where C_T is the threshold cycle. For immunohistochemical staining for human TSLP, human esophageal biopsies were embedded in paraffin and sectioned. Sections were deparaffinized and stained with a primary human TSLP-specific mAb or an isotype control antibody (validated by J.H. Yearley and R. de Waal Malefyt and commercially produced by Merck Research Laboratories), and positive staining was visualized using the DAB substrate kit (Vector Laboratories).

Flow cytometry. For mouse studies, esophageal tissues of two or three mice were pooled within each replicate experiment, opened longitudinally, digested in 1 mg mL^{-1} collagenase/DNase (Roche) for 30 min, and mashed through 70-µm nylon mesh filters. Single-cell suspensions were incubated with Aqua Live/Dead Fixable Dye (Invitrogen) for dead cell exclusion and stained with fluorochrome-conjugated mAbs purchased from eBioscience specific for mouse CD3ε (145-2C11, 1:300), CD4 (GK1.5, 1:300), CD8 (53-6.7, 1:300), NK1.1 (PK136, 1:300), CD19 (eBio1D3, 1:300), FcεR1 (MAR-1, 1:200), IgE (23G3, 1:200), CD45 (30-F11, 1:200), CD49b (DX5, 1:200,), CD117 (c-kit, 1:200), fluorochrome-conjugated mAbs purchased from Biolegend specific for mouse CD11c (N418, 1:200), CD5 (53-7.3, 1:300), B220 (RA3-6B2, 1:300) and a fluorochrome-conjugated mAb purchased from BD Bioscience specific for mouse Siglec-F (E50-2440, 1:200), or fluorochrome-conjugated mAbs purchased from eBioscience specific for human CD19 (HIB19, 1:200), CD45 (HI30, 1:100), CD49b (ebioY418, 1:200), FcεR1 (AER-37, 1:50), CD123 (6H6, 1:100) and c-kit (104D2, 1:30) or fluorochrome-conjugated mAbs purchased from BD Biosciences specific for human CD56 (B159, 1:200), CD11c (B-ly6, 1:200) and TCRαβ (IP26, 1:200). For intracellular staining, surface-stained cells were washed, fixed in 2% paraformaldehyde, permeabilized using eBioscience Permeabilization Buffer (eBioscience) according to manufacturer instructions, stained intracellularly with human 2D7-specific mAb (2D7, 1:25) (eBioscience), washed and

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resuspended in flow cytometry buffer. All cells were run on a four-laser 14-color LSR II (BD Biosciences), and FlowJo 8.7.1 (Tree Star) was used to analyze data. Mouse eosinophils were identified as live, lin$^-$ (CD3,CD5,CD19,CD11c,NK1.1), CD45$^+$Siglec-F$^+$ side-scatter (SSC)-high cells. Mouse basophils were identified as live, lin$^-$ (CD3,CD5,CD19,CD11c,NK1.1), c-kit$^-$CD49b$^+$IgE$^+$ cells (or as FcεRI$^+$ cells in *Igh-7*$^{-/-}$ mice). Human basophils in the esophageal biopsy were identified as live, lin$^-$ (CD19,CD56,CD11c,TCRαβ), CD49b$^+$FcεRI$^+$c-kit$^-$2D7$^+$ cells. Human basophils in the PBMCs were identified as live, lin$^-$ (CD19,CD56,CD11c,TCRαβ), CD123$^+$FcεRI$^+$ cells.

Optical coherence tomography. An OCT system operating at 1.3-μm center wavelength at 47 kHz axial scan rate (~30 frames per s) was developed and used for obtaining volumetric images of freshly excised mouse esophagus. The axial and transverse resolutions were 6 μm and 10 μm in tissue, respectively, and the imaging depth was approximately 2 mm, sufficient to image through the entire thickness of the mouse esophagus. Prior to OCT imaging, the esophagus was removed from the mouse, and a plastic tube with 0.75-mm outer diameter was inserted, allowing for the lumenal surface to be clearly differentiated in cross-sectional images. The esophagus was immersed in saline solution to remove light reflection from the surface. Subsequently, three-dimensional OCT images were obtained from multiple locations along the esophagus, with each data set covering 3 × 1.5 × 1.5 mm^3. The thickness values of the squamous epithelial layer were measured from cross-sectional OCT images every 200 μm along the esophagus within each data set. Average squamous epithelial thickness values from the middle of the esophagus were calculated from each mouse by an investigator blinded to group allocations and were used for comparison between different groups.

Mouse cell cultures, ELISA, real-time PCR, histology and electron microscopy. To measure spontaneous release of TSLP, whole ears were incubated for 12 h in complete culture medium (DMEM, 10% FBS), and cell-free supernatants were stored for a TSLP ELISA using a commercially available kit (eBioscience). For antigen re-stimulation, splenocytes or mesenteric lymph node cells were isolated, and single-cell suspensions were stimulated with 200 μg OVA for 72 h. Cell-free supernatants were used for standard sandwich ELISA. Antigen-specific IgE responses were measured as described previously[61]. For histological analysis, at necroscopy, the esophagus was fixed in 4% paraformaldehyde and embedded in paraffin, and 5-μm sections were cut and stained with hematoxylin and eosin (H&E). For immunofluorescence, sections were deparrafinized and stained with biotinylated Siglec-F–specific mAb from R&D Systems (BAF1706, 1:200), followed by secondary staining with Cy3-conjugated streptavidin (Jackson Laboratory) and counterstaining with DAPI (Molecular Probes). For EM, esophageal tissues were fixed with 2.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4 °C. After buffer washes, the samples were post-fixed in 2.0% osmium tetroxide for 1 h at room temperature and rinsed in dH_2O before *en bloc* staining with 2% uranyl acetate. After dehydration through a graded ethanol series, the tissue was infiltrated and embedded in Embed-812 (Electron Microscopy Sciences). Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage image capture software. For real-time PCR analysis, RNA was isolated from esophageal tissue using an RNeasy mini kit (Qiagen) or the mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. cDNA was generated using a SuperscriptII reverse transcription kit (Invitrogen). Real-time quantitative PCR was performed on cDNA using SYBR green master mix (Applied Biosystems) and commercially available primer sets from Qiagen (Quantitect primer assays). Samples were run on a real-time PCR system (ABI 7500; Applied Biosystems), normalized to β-actin and displayed as fold induction over controls.

Statistical analysis. Results are shown as mean ± s.e.m. To determine group sizes necessary for adequate statistical power, power analysis was performed using preliminary data sets for all analyses presented. Mice were assigned at random to treatment groups for all mouse studies. Mouse studies were not performed in a blinded fashion, except where indicated. Analyses of basophil responses in esophageal biopsy samples and peripheral blood were conducted in such a manner that the investigator was blinded to the disease state (number of eosinophils per HPF in the biopsy) and *TSLP* genotype until after flow cytometric analyses were completed. Analysis of *TSLP* expression levels in the biopsies of control subjects and those with EoE were not performed in a blinded fashion. All inclusion and exclusion criteria for mouse and human studies were pre-established. For mouse studies, statistical significance was determined using a nonparametric, two-tailed Mann-Whitney *t*-test, a nonparametric, one-way Kruskal-Wallis ANOVA test followed by Dunn's *post hoc* testing or a nonparametric, two-way ANOVA followed by Bonferroni's *post hoc* testing. For human studies, a nonparametric, two-tailed Mann-Whitney *t*-test or a nonparametric, one-way Kruskal-Wallis ANOVA followed by Dunn's *post hoc* testing were used. Correlation analysis was performed using a nonparametric Spearman correlation (sensitivity analyses were performed), and a linear regression of the data is displayed. All data meet the assumptions of the statistical tests used. Within each group there is an estimate of variation, and the variance between groups is similar. For each statistical analysis, appropriate tests were selected based on whether the data was normally distributed and whether multiple comparisons were made. Results were considered significant at $P \leq 0.05$. Statistical analyses were performed using Prism version 5.0a (GraphPad Software).

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